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Conservation biology of the barbastelle (Barbastella barbastellus) : applications of spatial modelling, ecology and molecular analysis of diet

CONSERVATION BIOLOGY OF THE BARBASTELLE (*BARBASTELLA BARBASTELLUS*)

APPLICATIONS OF SPATIAL MODELLING, ECOLOGY AND MOLECULAR ANALYSIS OF DIET

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Abstract

This study uses a multidisciplinary approach to fill current knowledge gaps in the ecology and conservation requirements of a rare bat, the barbastelle (*Barbastella barbastellus*). Spatial modelling was used to evaluate the environmental parameters that dictate where the species is able to persist in the UK and to identify areas of high conservation value. Models indicated that *B. barbastellus* is highly dependent on large areas of native mature woodland and is limited in its distribution predominantly by summer climate, although a number of other environmental factors also appear to be important. Ground-validation of model predictions resulted in the discovery of three new maternity colonies. Radio-tracking was used to investigate roost preferences, home range use and foraging habitat requirements. Preferences for roosting in old or dead oak (*Quercus* spp.) trees were identified, although potentially any tree supporting a suitable roost cavity may be used. Trees located near to rivers or streams within unmanaged broadleaved woodland were favoured. Bats selected riparian and broadleaved woodland habitats above all others for foraging, presumably to maximise encounter rates with preferred moth prey. Unimproved grassland and field boundary features were also important components of the foraging environment. The range spans and home range areas of individual bats varied enormously. Although the average range span of colonial females was 8 km, a few bats travelled up to 20 km to reach core foraging areas. Colony members showed distinct spatial organisation, with minimal overlap of foraging areas and high fidelity to foraging sites. A novel molecular technique for diet analysis based on DNA barcoding was designed and tested to improve the detection and resolution of prey within the faeces of insectivorous bats. The sensitivity and taxonomic resolution of the molecular method was far superior to conventional morphological techniques. Prey items are now readily identifiable at the species level, providing new perspectives on the dietary requirements and trophic ecology of bats. Both DNA-based and morphological techniques were used to determine the diet of *B. barbastellus*. In total, eighty-nine different prey items were identified and nearly all were resolved to species. The results showed that *B. barbastellus* feeds almost exclusively on moths that have ears, prompting questions about how the species is able to bypass the hearing defences of prey. The implications of all the findings for *B. barbastellus* conservation are discussed and management recommendations are made.

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Matthew Richard Kemish Zeale

SIGNED: DATE:

TABLE OF CONTENTS

List of Tables and Figures

CHAPTER 1

General Introduction

1.1 Conservation ecology of bats

Bats (Order Chiroptera) are the second most diverse mammal group, comprising some 1100 extant species (Simmons 2005). They represent nearly a quarter of all mammal species and occupy a broad range of biomes and habitats on all continents except Antarctica. The considerable numerical and ecological diversity found among bats and the numerous specialised characteristics that they possess – e.g. powered flight, echolocation, hibernation, high niche specialisation – make them exceptional models for studying many evolutionary and ecological principles. Because bats operate at high trophic levels they have a significant influence on coexisting species and the overall structure and functionality of ecosystems. Within their respective biological communities bats perform a wide variety of important ecosystem services, from key pollinators and seed dispersers in the tropics to natural control agents of arthropod populations in temperate regions (Nowak 1994). Many of these services also have benefits for humans including *inter alia* facilitating agricultural production (Williams-Guillén *et al.* 2008; Kalka *et al.* 2008) and disease control (Reiskind & Wund 2009).

Nearly a quarter of extant bat species are listed as threatened under IUCN (International Union for Conservation of Nature) criteria and many more are in decline (Hutson *et al.* 2001; Racey & Entwistle 2003). As is true for biodiversity in general, anthropogenic stressors, including habitat modification, pesticide use, overhunting, and persecution, are considered to be primary causal factors for current population declines (Racey & Entwistle 2003). Collisions with wind turbines and road kills also appear to be increasing pressures on bat populations (Kunz *et al.* 2007; Arnett *et al.* 2008). Crucially, bats possess a number of life history traits that render them particularly vulnerable to negative environmental change, such as slow population growth, high ecological niche specialisation, and high trophic status. Many species also demonstrate natural rarity and genetic isolation (Racey & Entwistle 2003). Given their high sensitivity to environmental change, bats have enormous potential as bioindicators (Jones *et al.* 2009).

Addressing current population trends in bats is a major challenge for conservationists. Action plans developed by the IUCN Chiroptera Specialist Group have been progressive in highlighting the conservation status of bats (Mickleburgh *et al.* 1992; Hutson *et al.* 2001; Mickleburgh *et al.* 2002); however, for many species too few data are available to determine their specific conservation needs. Obtaining information on the ecological

requirements and spatial distributions of bats is fundamental to evaluating the threat to species and for providing a platform from which effective management decisions can be made. Given the multitude of intrinsic (e.g. ecological specialisation, distribution, rarity) and extrinsic (e.g. habitat availability, prey availability, climatic conditions) factors that determine a species' biology, successful conservation of bats will depend inherently on the integration of expertise and techniques from a broad range of scientific disciplines.

1.2 Integrated approaches to conservation

'Integrative biology' is a term used to describe methods of research that draw upon a variety of scientific disciplines to answer complex biological questions (Wake 2001; 2003). Integrated approaches typically involve cross-disciplinary cooperation (i.e. bringing together researchers of diverse expertise to identify, articulate, and structure problems) as well as hierarchical explorations of the issue (e.g. using observational, experimental and modelling approaches) (Wake 2008). Recent examples where integrated approaches have been used to study bats include the use of bioacoustics, behaviour and molecular techniques to identify cryptic species (Jones & van Parijs 1993; Barratt *et al.* 1997) and to define their distinct ecology (Davidson-Watts & Jones 2006; Davidson-Watts *et al.* 2006), the use of bat flight path tracking, moth neurophysiology and molecular analysis of diet to investigate coevolution in predator-prey relationships (Goerlitz *et al.* 2010), and combining phylogenetic data with past and present predictive modelling to determine population structures (Rebelo 2009).

The concept of integrative biology is one that is central to species conservation. The most successful conservation strategies are likely to be those that are founded on the broadest understanding of a species' complete biology and life history. Acquiring this understanding requires the integration of a broad range of disciplines and techniques to investigate species at all levels of biological complexity (e.g. genes, organisms, populations, ecosystems). In many cases this may necessitate the development of new techniques and methodologies, embracing new technologies and advances in theory as and when they manifest. Given that researchers are becoming increasingly more specialised in their fields of expertise, tackling biodiversity loss in the future will demand increasing levels of cooperation and collaboration throughout the scientific community.

1.3 *Barbastella barbastellus*

The barbastelle, *Barbastella barbastellus* (Schreber, 1774) is a medium-sized insectivorous bat (forearm: 36.5–43.5 mm, weight: 6–13.5 g; Schober & Grimmberger 1997; Russo *et al.* 2004) of the family Vespertilionidae. The species occurs in Europe, North Africa and Asia and is morphologically distinct from sympatric species by its dark black-brown colouration and broad trapezium-shaped ears that join across the forehead (Rydell & Bogdanowicz 1997; Dietz *et al.* 2009). In the eastern Caucasus where the species' range overlaps with that of the congeneric eastern barbastelle, *Barbastella leucomelas*, measurable differences in body size and facial features distinguish the two species. *B. barbastellus* is considered one of the rarest and most threatened bat species in Europe (Stebbings 1988; Urbańczyk 1999). In many countries where it is present, the species is legally listed as 'Vulnerable' or 'Endangered' (Stebbings 1988; Bulgarini *et al.* 1998). Although initially described as 'Vulnerable' at the global scale (1996), following a status review in 2008 the species is now listed as 'Near Threatened', although populations are still considered to be declining (IUCN Red List of Threatened Animals; http://www.iucnredlist.org/apps/redlist/details/2553/0). Congregations of hundreds or thousands of individuals within underground hibernation sites in eastern Europe are the exception to an otherwise characteristically sparse distribution (Uhrin 1995; Gottfried 2009); typically the species occurs in low densities and numbers (Rydell & Bogdanowicz 1997). Populations throughout Europe have shown dramatic declines over the past century (Hutson *et al.* 2001), primarily due to the loss and fragmentation of native mature woodland habitats, on which the species is heavily dependent for roosting (Greenaway 2001; Russo *et al.* 2004, 2005) and foraging (Sierro 1999; Greenaway 2001). The general trend towards habitat simplification and increased use of pesticides associated with agricultural intensification, and disturbance or loss of underground sites, are also thought to have contributed significantly to declines.

B. barbastellus exhibits high ecological specialisation and thus is likely to be highly sensitive to negative changes in its environment. The species has one of the narrowest diets among Palaearctic bat species (Rydell *et al.* 1996; Sierro & Arlettaz 1997) and shows specific foraging and roosting habitat requirements (Sierro 1999; Russo *et al.* 2004). Prior to this study (2006), *B. barbastellus* was an understudied and little known species, particularly in the UK where only one study of the species had been conducted

(Greenaway 2001). In the past six years, however, *B. barbastellus* has received much attention throughout Europe. Studies on roosting ecology in Italy (Russo *et al.* 2004, 2005, 2010), spatial organisation and site fidelity in Germany (Hillen *et al.* 2009, 2010), the effects of roads on commuting bats, also in Germany (Kerth & Melber 2009), hibernation in Slovakia (Gottfried 2009), modelled distributions in Portugal (Rebelo & Jones 2010), and predicted responses to climate change (Rebelo *et al.* 2010), have all contributed to better understanding the species' ecological requirements and the threats it faces from human activities; however, critical knowledge gaps remain, most notably in areas relating to foraging ecology and diet and associated habitat requirements.

B. barbastellus is protected by European law under Appendix II of the Bonn Convention (and its Agreement on the Conservation of Bats in Europe, 1994), Appendix II of the Bern Convention (and its appropriate recommendations) and Annexes II and IV of the EC Habitats and Species Directive. In the UK, where possibly only 5,000 individuals persist (Harris *et al.* 1995; Richardson 2000), the species is protected under Schedule 2 of the Conservation Regulations (1994), Schedule 5 of the Wildlife and Countryside Act (1981) and is a UK Biodiversity Action Plan (UK BAP) species.

1.4 Purpose of this research

Foremost, this study intends to fill current knowledge gaps in the ecology and conserve-ation requirements of *B. barbastellus*. In doing so, key research objectives within the *B. barbastellus* Species Action Plan (SAP; Anon 1998) will be addressed. To achieve this, a multidisciplinary approach is used, involving the integration of spatial modelling, field-based ecology, and molecular approaches to diet analysis. The specific aims of this study are five-fold:

- to use species distribution modelling techniques to (a) estimate the current distribution of *B. barbastellus* in the UK and identify the environmental parameters that dictate where the species is able to persist, and (b) to evaluate the merits of these techniques in facilitating the identification of areas of high conservation value.

- to determine the roosting requirements of the species by assessing roost selection on three levels: woodland structure and management type, tree characteristics, and cavity characteristics.

- to investigate the home range use, foraging habitat selection and facets of foraging behaviour such as spatial organisation and foraging site fidelity to determine the landscape-scale habitat requirements of breeding populations.

- to design and test an efficient, non-invasive molecular tool for making robust species-level identifications of arthropod prey in the diets of insectivorous bats, and thus provide a method that offers much enhanced understanding of bat dietary ecology.

- to investigate in unprecedented detail the diet and trophic ecology of *B. barbastellus* through the application of both DNA-based and conventional morphological approaches to faecal analysis.

The implications of all the findings for *B. barbastellus* conservation are discussed and management recommendations are made.

1.5 Thesis outline

Each chapter in this thesis is dedicated to fulfilling one of the five study aims mentioned above. First, Chapter 2 investigates the merits of presence-only modelling techniques for facilitating conservation planning. The environmental parameters that dictate the distribution of *B. barbastellus* in the UK are examined and model predictions are used to facilitate the discovery of new maternity colonies.

Having examined the relative importance of the broad-scale environmental parameters that govern habitat suitability, in Chapter 3 the specific features of woodlands that make them favourable as roosting areas is investigated. Roost preferences of radiotracked bats are examined by comparing the characteristics of roost trees and roost cavities with those of random trees and cavities. I test the hypothesis that roost selection is random at all levels. The results are used to make recommendations for woodland management.

In Chapter 4, bats are radiotracked beyond woodland roosting areas to investigate home range use and habitat selection by foraging bats. I test the hypotheses that (a) individual bats forage spatially at random within home ranges and (b) foraging bats use habitat types in proportion to their availability. The importance of different habitat types

to bats is discussed and landscape-scale management recommendations are made that, if implemented, will serve to promote foraging opportunities for *B. barbastellus*.

In Chapter 5, a novel molecular methodology for making species-level identifications of arthropod prey in bat faeces is presented. The performance of the methodology is tested empirically using faecal samples from three bat species that specialise in eating different types of insect prey and the value of the method for improving understanding of bat dietary ecology is discussed.

In Chapter 6, a dietary study is undertaken, combining the molecular methodology described in Chapter 5 with a conventional morphological approach to examine in unprecedented detail the diet and trophic ecology of *B. barbastellus*. Hypotheses about prey selection and prey defences are examined and new perspectives on diet composition are discussed with regard to implications for conservation.

Chapter 7 is a general discussion, whereby all the findings from the previous chapters are summarised and related to one another. Final conclusions are made, particularly in reference to the conservation of *B. barbastellus*.

CHAPTER 2

Identification of maternity colonies using ground validation of presence-only modelling

Abstract

For rare bat species, the identification and protection of maternity colonies is an essential part of their conservation management. In this study, I investigate the value of using species distribution modelling (Maxent) for facilitating the identification of *Barbastella barbastellus* maternity colonies within woodland areas. For this purpose, two models were developed. First, *B. barbastellus* species distribution was modelled at the UK-wide scale to identify suitable regions in which to conduct searches for maternity colonies. Then, a second model was developed to predict the species' presence or absence within woodlands throughout the chosen study area (south-west Wales). The predictions made by the latter model were ground-validated by surveying 30 woodlands for *B. barbastellus* presence using acoustic monitoring and trapping methods. Analysis of the model's performance showed that it predicted *B. barbastellus* presence/absence to a high degree of accuracy. The species was recorded in nine woodland sites and one or more bats were caught in three sites. Subsequent radiotracking of captured bats to day roosts resulted in the identification of three new maternity colonies. Fundamentally, the results reinforce the need to preserve large areas of native mature woodland for effective conservation of *B. barbastellus*. This study shows that presence-only modelling, when integrated with conventional field survey techniques, can be used to great effect to aid the identification of *B. barbastellus* maternity colonies. Accordingly, the use of this technique in future species conservation planning is highly recommended.

2.1 Introduction

Determining the distribution of rare species is a key component of their conservation management (Racey & Entwistle 2003). However, for elusive animals such as bats, difficulties associated with their detection in the field place important constraints on the accumulation of reliable presence data. Developments in geographical information systems (GIS) and multivariate modelling techniques have provided a platform for making accurate predictions about species distributions (Jaberg & Guisan 2001; Wang *et al.* 2003) and offer a more focussed approach to species detection in the field by identifying areas of high suitability (Segurado & Araújo 2004; Rebelo & Jones 2010).

A variety of species distribution modelling techniques have been developed to date (reviewed in Franklin 2009) that seek to define associations of study species with spatial environmental variables (e.g. habitat type, mean temperature, altitude) using either presence and absence, or presence-only data to train models. For bats, models that require presence-only datasets are favourable because true absences are difficult to confirm. Of the presence-only models currently available – including for example, Bioclim (Busby 1986), Domain (Carpenter *et al.* 1993), Genetic Algorithm for Rule-set Prediction (GARP; Stockwell & Peters 1999) Ecological Niche Factor Analysis (ENFA; Hirzel *et al.* 2002), and Maximum Entropy modelling (Maxent; Phillips *et al.* 2006) – Maxent has proven to be one of the most successful at predicting a species' realised niche (Hernandez *et al.* 2006; Pearson *et al.* 2007; Wisz *et al.* 2008) and remains sensitive even when only few training data are available (Wisz *et al.* 2008; Rebelo & Jones 2010); a feature that is particularly advantageous for rare species where presence records may be scarce.

Previous studies have shown that Maxent performs well when predicting areas of high suitability for *Barbastella barbastellus* over large spatial scales (Rebelo & Jones 2010). Rebelo & Jones (2010) discovered 15 new *B. barbastellus* sites and extended the known distribution of the species in Portugal by approximately 100 km. Here, I use Maxent to develop habitat suitability maps for *B. barbastellus* in the UK and use model predictions to aid the discovery of maternity colonies in south-west Wales where, prior to this study, only one colony record existed. For bats, maternity colonies are arguably the unit of greatest conservation value, yet for woodland-dwelling species, including *B. barbastellus*, the identification of maternity colonies can be problematic. Usually, radiotracking is required to locate bats to specific roost cavities from which initial estimates of colony size

can be made; hence, identifying woodlands of high suitability for maternity colonies – where densities of the study species will be high – is useful for focusing resources toward areas in which the probability of successful captures is highest. For *B. barbastellus* which echolocates at low intensities (Goerlitz *et al.* 2010), and therefore is detected less well by conventional acoustic survey methods than other sympatric bat species, such an approach may significantly improve the efficiency with which woodland areas of high conservation value can be identified.

In this study, two models are developed. First (Model 1), *B. barbastellus* distribution in the UK is modelled to identify regions of high suitability that can be targeted as appropriate study areas in which to conduct searches for maternity colonies. This model also provides an evaluation of the environmental parameters that dictate where *B. barbastellus* populations can persist in the UK. Second (Model 2), species distribution is modelled again but on a comparatively smaller spatial scale to identify specific woodlands of high suitability for *B. barbastellus* within the chosen study area. The predictions of this model are used to focus field resources towards successful captures of bats for radiotracking. In addition, the accuracy of the predictions is tested by conducting ground validation surveys for *B. barbastellus* presence/absence within woodlands of both high and low predicted suitability. Because the main aim of this study is to evaluate the use of presence-only modelling for identifying maternity colonies, analyses and discussions are focussed primarily on the performance of Model 2. All the work relating to Model 2 is my own, however, it should be noted that Model 1 was developed by Orly Razgour (University of Bristol, Bristol, UK; unpubl. data) and is replicated here with permission. The output of Model 1 is essential however to set the context for the detailed field studies performed to test predictions generated by Model 2. For clarity, the methods and results for each model are presented separately.

Currently, fewer than 20 *B. barbastellus* maternity colonies have been confirmed in the UK. Locating new colonies is therefore considered a major conservation priority and is a key objective within the Species Action Plan for this species (SAP; Anon 1998). The main objectives of this study were therefore: (i) to identify new *B. barbastellus* maternity colonies not previously recorded (so that they may be afforded protection), and (ii) to evaluate the extent to which Maxent can serve as a catalyst for accelerating the identification of suitable sites for this rare species.

2.2 Materials and methods – Model 1

2.2.1 Modelling procedure

The Maxent modelling technique was chosen for both models (Model 1 and Model 2) over other presence-only modelling techniques for its comparatively high performance (Elith *et al.* 2006; Hernandez *et al.* 2006) and for its noted retention of high predictive success when only limited presence training data are available. Maxent is a machine learning-process that applies a maximum entropy principle to estimating a species' probability distribution, subject to the constraint that the expected value of each environmental variable (e.g. altitude, temperature, land cover) should match the empirical average of the presence data (locations of known species occurrence). To provide an assessment of which environmental variables are most important to model performance (i.e. the most informative predictors of species presence), Maxent performs a Jackknife analysis of the effect of each variable on overall gain (a measure of how much better the modelled distribution fits sampled points than does the uniform distribution). For this analysis, a series of models are generated to provide two measures of 'importance' for each variable. First, models are generated using each variable in isolation to determine which variable has the most useful information by itself. Then, each variable is excluded in turn and a model generated using all the remaining variables to determine which variable contributes the most information that is not present in the other variables, i.e. the most uncorrelated information (measured as the reduction in gain). The overall contribution of each variable to the model is also provided by Maxent. For each iteration of the training algorithm (n = 1000 iterations) the increase in regularized gain is added to the contribution of the corresponding variable and the accumulated scores are regularized to give the relative percent contribution of each variable.

2.2.2 Model building

Model 1 was developed to identify areas of high suitability for *B. barbastellus* on a UK-wide scale. For model calculation, all historical presence records (including all acoustic, trapping and roost records) for *B. barbastellus* in the UK (n = 92) up to 2008 were obtained from the National Biodiversity Network (NBN) Gateway website (http://data.nbn.org.uk/) (Fig 2.1). In addition, a set of independent environmental variables were

selected as predictors of species presence. These included land cover (source Global Land Cover 2000; http://www-gvm.jrc.it/glc2000/), geology (source British Geological Survey; http://www.bgs.ac.uk/products/home.html), artificial night light (to which some species of bat are known to be highly sensitive (Stone *et al.* 2009); source National Oceanic and Atmospheric Administration; http://www.ngdc.noaa.gov/dmsp/), human population density (included as a representation of anthropogenic disturbance; source Ridge National Laboratory; http://www.ornl.gov/sci/landscan/), and a number of climatic variables (source WorldClim; http://www.worldclim.org) including various derivations of temperature and precipitation, both of which are known to have a great relevance to bat physiology and survival (Altringham 1996). A description of each environmental variable used in model calculations is provided in Table 2.1. All digital information, including presence locations, had a resolution of ~1 km. Data manipulation and formatting of environmental variables was performed in ArcGIS 9.2 (Esri Inc., Redlands, CA, USA) and all model calculations were performed in Maxent v3.3.3a (http://www.cs.princeton.edu/~schapire/maxent/).

Table 2.1 – Description of environmental variables used as predictors of species presence in Maxent model calculations of *B. barbastellus* distribution in the UK (Model 1).

Variable	Type	Description
Altitude	Continuous	Elevation above sea level (m)
Geology	Categorical	Classification of bedrock geology (n = 22 categories; refer to Appendix 1).
Land cover	Categorical	Classification of land cover habitat types (n = 9 categories; refer to Appendix 1).
Human population density	Continuous	Density of human population, scored as the absolute number of inhabitants per unit area (resolution 30 arc seconds); as recorded in 2008.
Night light	Continuous	Night-time anthropogenic light pollution, scored as normalised pixel-based units of luminosity from satellite digital imagery; as recorded in 2008.
Total annual rainfall	Continuous	Total rainfall per annum (mm).
Total summer rainfall	Continuous	Total rainfall during the warmest annual quarter (mm).
January maximum temperature	Continuous	Absolute maximum temperature for January (°C).
Spring mean temperature	Continuous	Mean temperature during March-May (°C).
Summer mean temperature	Continuous	Mean temperature during the warmest annual quarter (°C).
Annual temperature range	Continuous	Range between absolute maximum and absolute minimum temperatures per annum (°C).

2.2.3 Model evaluation

To evaluate model performance, 25 % of the training data (presence records) were used to test the threshold-independent (scaled probability of presence from 0 (absence) to 1 (presence)) projections of model predictions. The area under the curve (AUC) of the Receiver Operating Characteristics (ROC) plot was used as a single measure of model performance (Fielding & Bell 1997). The ROC plot is generated by plotting sensitivity (accuracy in predicting presence) against 1–specificity (inverse accuracy in predicting absence); hence, the AUC is the probability that the model correctly ranks a random presence site versus a random absence site. Values for the AUC range from 0.5 (complete randomness) to 1 (perfect discrimination). To identify which of the environmental variables used as predictors of species presence were most informative, the Maxent-generated Jackknife analysis and the calculations of percent contribution of each variable to the model were examined.

2.3 Results – Model 1

2.3.1 Model evaluation

The modelled habitat suitability map for *B. barbastellus* in the UK is displayed in Fig. 2.1. Examination of the model ROC plot revealed a high AUC score (AUC = 0.904), indicating that the model possessed high predictive ability. An inspection of the Jackknife analysis showed that mean summer temperature and summer rainfall were the environmental variables that contributed most to the model and hence were the most informative predictors of *B. barbastellus* presence at the UK-wide scale (Fig. 2.2). The variable with highest gain when used in isolation was mean summer temperature, which therefore appears to have the most useful information by itself. The variable that decreased the gain the most when it was omitted was summer rainfall, which therefore appears to have the most information that is not present in the other variables. The model predicted probability of *B. barbastellus* presence to be most strongly associated with mean summer temperatures above approximately 15 °C (mean 24 hour temperature) and where the total summer rainfall does not exceed 250–300 mm ('summer' refers to the warmest annual quarter). The species is predicted not to be able to persist where the mean

summer temperature is below 12 °C. Among the other variables examined, human population density, maximum January temperatures, geology, land cover, and altitude also appear to be informative predictors of *B. barbastellus* presence (Fig. 2.2). Presence is predicted to be most strongly associated with low human population densities, mean January temperatures above 6 °C, chalk bedrock, broad-leaved woodland habitat, and altitudes below approximately 400 m. The remaining environ-mental variables contributed comparatively little to the model (Fig. 2.2). Individual probability of presence plots (from which the above values are extracted) for each of the environmental variables used in model calculations are provided in Appendix 1.

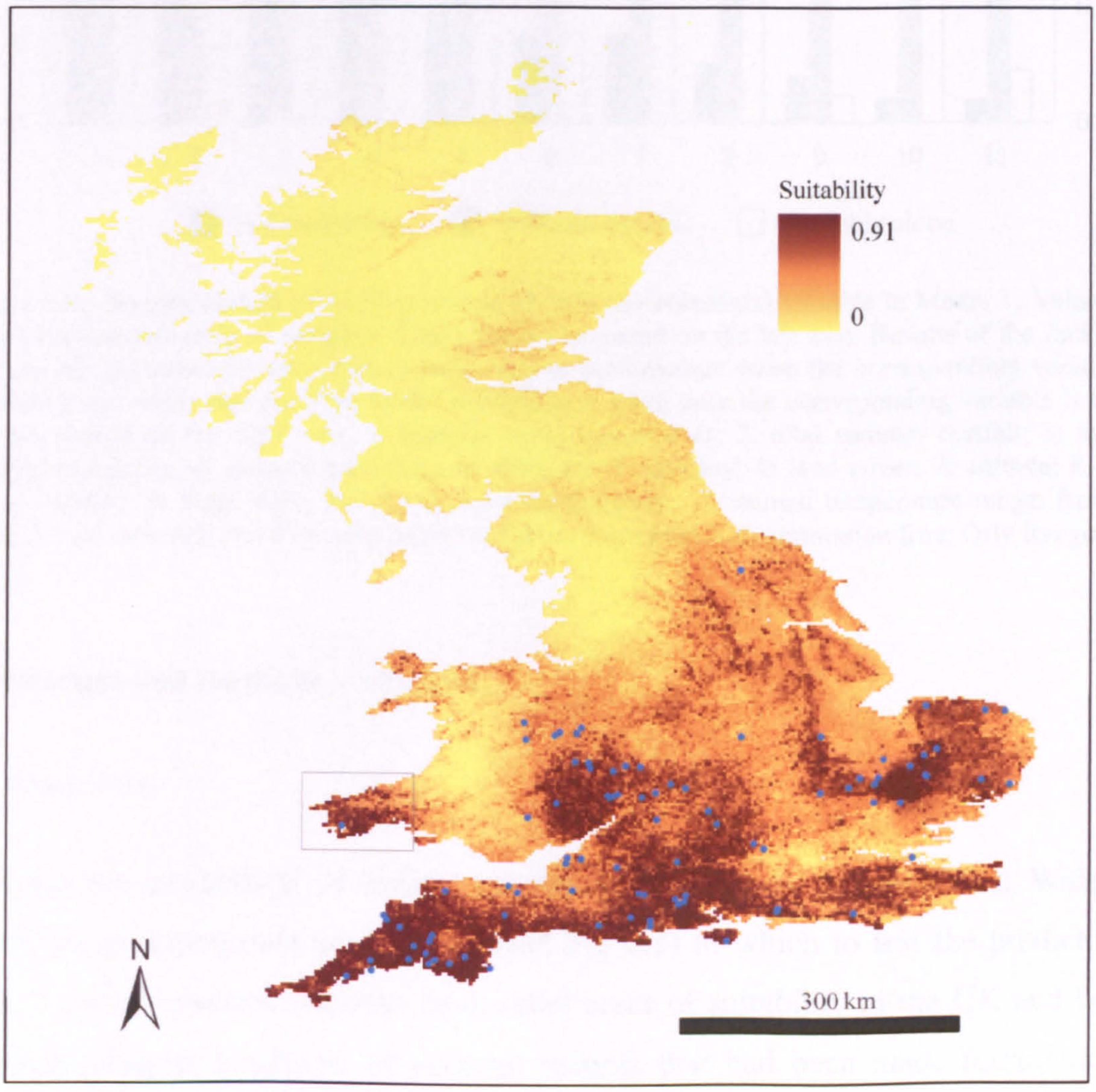

Figure 2.1 – Habitat suitability map for *B. barbastellus* in the UK, as calculated by Maxent using the environmental variables listed in Table 2.1 and presence training data (blue points) obtained from the NBN Gateway website; replicated with permission from Orly Razgour, University of Bristol, UK. Inset: study area within which searches for maternity colonies were conducted based on the predictions of Model 2.

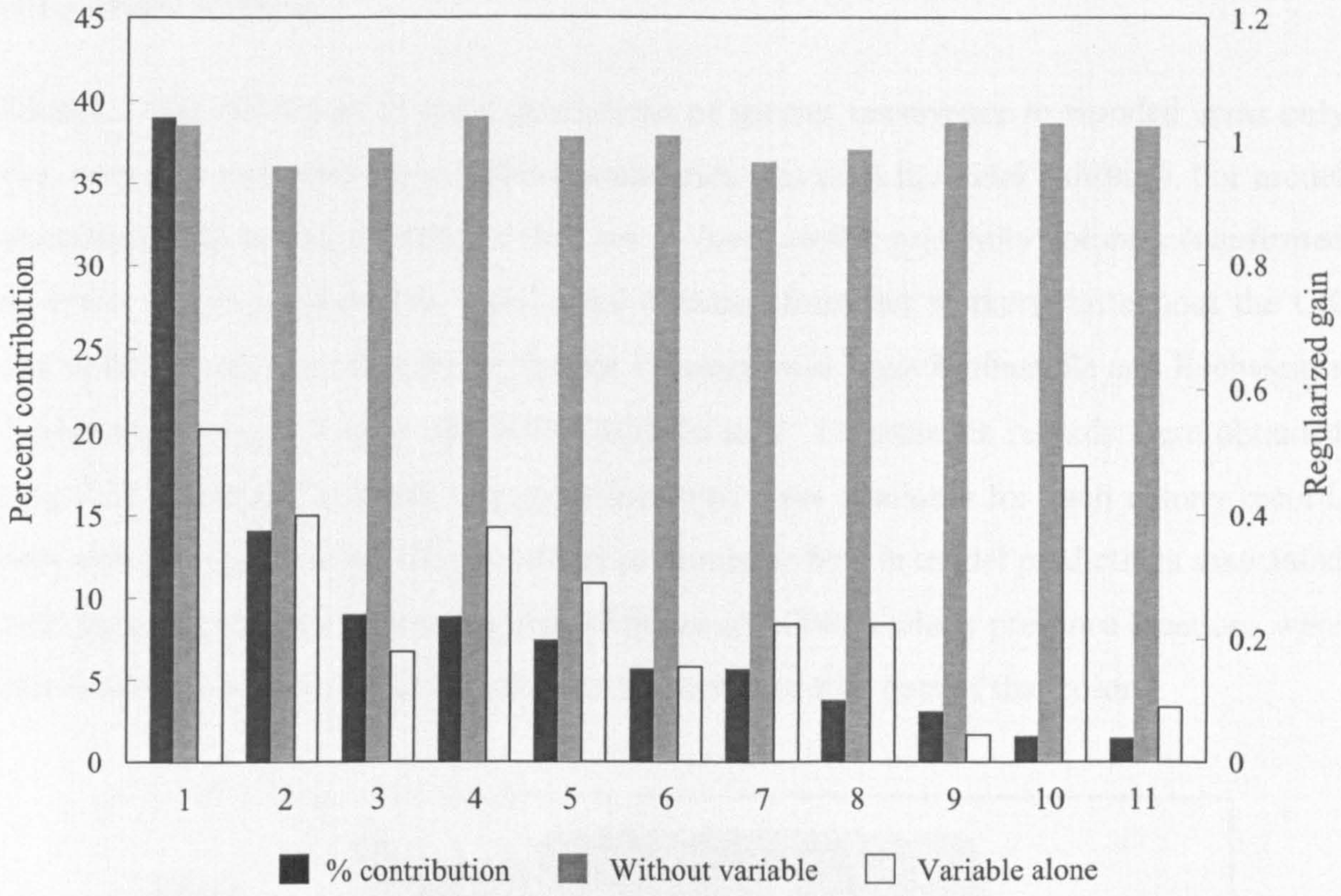

Figure 2.2 – Representation of the importance of each environmental variable to Model 1. Values for the percent contribution of variables (black bars) are scored on the left axis. Results of the Jackknife analysis are portrayed by grey bars (relative model performance when the corresponding variable is excluded) and white bars (relative model performance when only the corresponding variable is used) and are scored on the right axis. 1: summer mean temperature; 2: total summer rainfall; 3: human population density; 4: January maximum temperature; 5: geology; 6: land cover; 7: altitude; 8: total annual rainfall; 9: night light; 10: spring mean temperature; 11: annual temperature range. Refer to Table 2.1 for descriptions of environmental variables. Presented with permission from Orly Razgour.

2.4 Materials and methods – Model 2

2.4.1 Study area

Following the predictions of habitat suitability from Model 1, south-west Wales was selected as an appropriate study area (inset Fig 2.1) in which to test the predictions of Model 2 for its apparent isolation from other areas of suitability in the UK and because previously, despite a number of acoustic records that had been made there (Margaret Clarke, unpubl. data), only one *B. barbastellus* maternity colony had been confirmed in the area. The dominant land cover in the study area is pastoral farmland and although woodland is present throughout (predominantly within the extensive valley networks that characterise the area) relatively few areas of ancient native woodland persist. Managed conifer plantations are present mainly as large isolated woodland blocks.

2.4.2 Model building

Model 2 was developed to focus predictions of species occurrence to wooded areas only (i.e. only data delimited by woodland boundaries was used in model building). For model calculation, all available presence data for *B. barbastellus* maternity colonies (confirmed as active within the last five years) were obtained from bat workers throughout the UK and collated with records held by the Bat Conservation Trust Barbastelle and Bechstein's Technical Advisory Group (BCT/BBTAG). In total, 18 presence records were obtained (Fig 2.3). Although multiple tree roost locations were available for each colony record, only one presence was scored per colony to minimise bias in model predictions associated with pseudoreplication of training data (Elith *et al*. 2006). Colony presence locations were calculated as the mean centre of all roost locations used by bats of that colony.

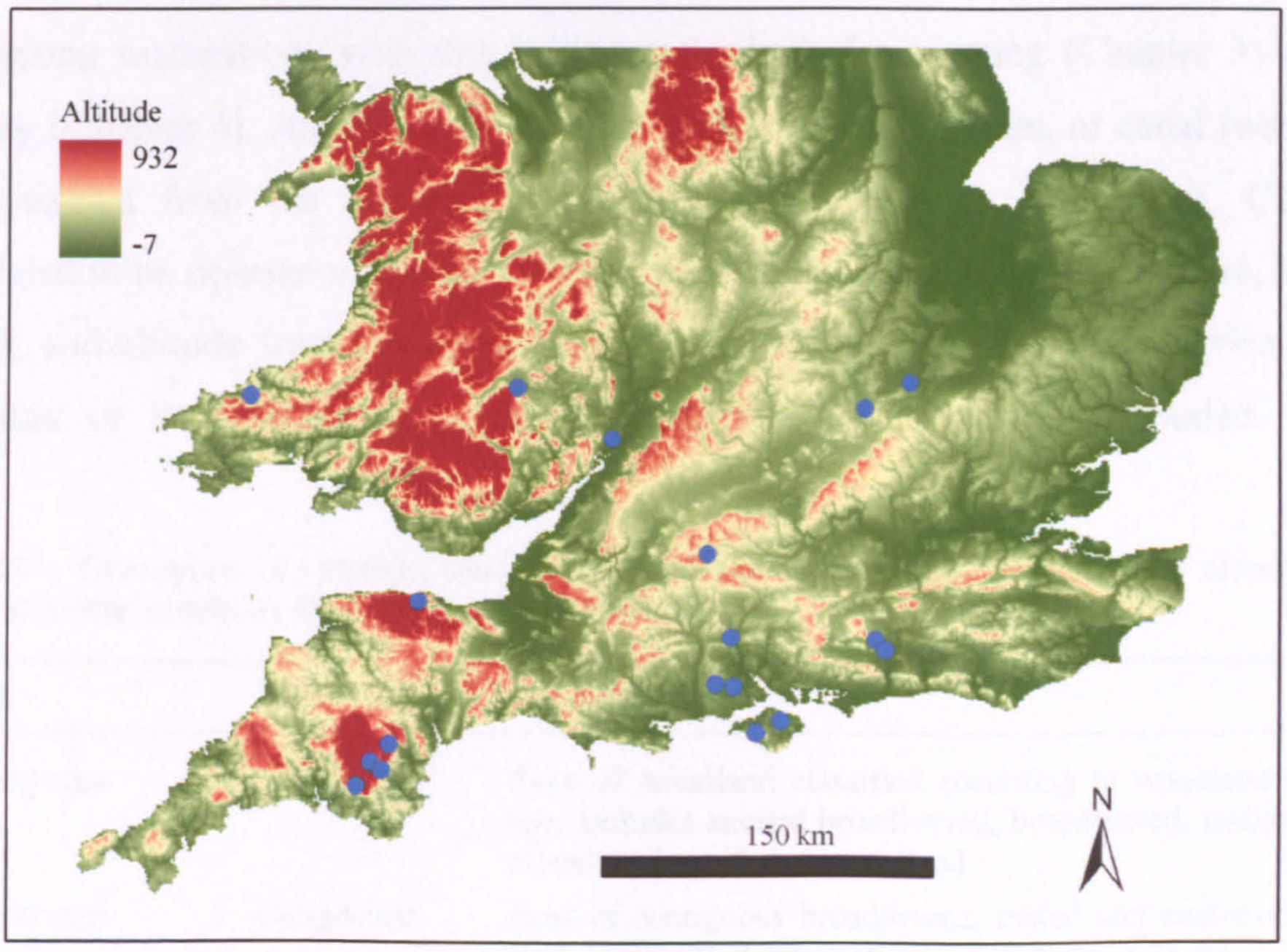

Figure 2.3 – *B. barbastellus* maternity colony presence records used as training data for Model 2.

The selection of environmental variables focussed predominantly on woodland characteristics (Table 2.2). Three sources of data were used to classify woodlands by type (national inventory of woodlands and trees, sourced from the Forestry Commission, Leicester, UK) and age (Ancient Woodland Inventory (England), sourced from Natural England (www.naturalengland.org.uk); ancient and semi-natural woodland dataset

(Wales), sourced from the Countryside Council for Wales, Bangor, Wales). Because *B. barbastellus* colonies have been shown to require relatively large woodland areas for roosting (Greenaway 2001; Russo *et al.* 2004, 2005; Chapter 3), 'woodland size' (delimited by woodland type) and 'density of broadleaved woodland' were also extracted from the above datasets and used in model calculations. Woodland size was reclassified into four categories (Table 2.2) determined by four natural breaks in the distribution (as calculated by ArcGIS 9.2). Density of woodland was considered important because size alone may be a misleading interpretation of woodland suitability; depending on the extent of fragmentation, clusters of smaller woodlands may be of equal suitability as one large contiguous area. Given that the maximum distance between tree roosts used by a single colony rarely exceeds 1-2 km (Russo *et al.* 2004; Chapter 3), an area of 1 km radius was considered appropriate for calculations of woodland density. 'Density of riparian woodland' was also included in the model because *B. barbastellus* has been shown to have strong associations with this habitat type, both for roosting (Chapter 3) and for foraging (Chapter 4). Any woodland within 30 m of a river, stream, or canal (waterways data obtained from the Centre for Ecology and Hydrology, Edinburgh, UK) was considered to be riparian woodland. Finally, because mean summer temperature, summer rainfall, and altitude were previously identified by Model 1 as important environmental predictors of *B. barbastellus* presence, these variables were also included. Human

Table 2.2 – Description of variables used as environmental predictors in Maxent model calculations of woodland habitat suitability for *B. barbastellus* (Model 2).

Variable	Type	Description
Woodland type	Categorical	Type of woodland classified according to woodland type and age; includes ancient broadleaved, broadleaved, ancient mixed, mixed, and coniferous woodland.
Woodland size	Categorical	Area of contiguous broadleaved, mixed and coniferous woodland, reclassified into categories: 0-4 ha, 4-38 ha, 38-312 ha, and >312 ha.
Density of broadleaved woodland	Continuous	Density of broadleaved woodland within an area delimited by 1 km radius.
Density of riparian woodland	Continuous	Density of riparian woodland (woodland within 30 m of a river, stream or canal) within an area delimited by 1 km radius.
Summer mean temperature	Continuous	Mean temperature during the warmest annual quarter (°C).
Summer rainfall	Continuous	Total rainfall during the warmest annual quarter (mm).
Altitude	Continuous	Elevation above sea level (m).

population density and geology, which were also identified as informative variables by Model 1 (Fig. 2.2), were not included due to the restrictions of limited training data (n = 18) on the total number of variables that can be used to build models. Moreover, human population density was not considered informative because woodland areas typically are uninhabited. To retain good spatial resolution of woodland boundaries, all digital information was scaled to 50 m pixels. All data manipulation and formatting of environmental variables was performed in ArcGIS 9.2 and all model calculations were performed in Maxent v3.3.3a.

2.4.3 Model evaluation

Initially, predictions of species distributions calculated by Maxent are provided on a continuous (threshold independent) probability scale ranging from 0 (absence) to 1 (presence). Therefore, to generate a binary (threshold dependent) presence/absence map of the study area – so that woodlands could be classified as suitable (predicted *B. barbastellus* presence) or unsuitable (predicted absence) – the 10^{th} percentile presence value (above which it is considered that the species will be present) was used as a threshold to discriminate suitable woodlands from unsuitable woodlands (Phillips *et al.* 2006; Raes *et al.* 2009). To evaluate model accuracy and performance, both threshold-independent and threshold-dependent projections of model predictions were compared with test data. For the threshold-independent analysis, 25% of the model training data were used as test data and the area under the curve (AUC) of the Receiver Operating Characteristics (ROC) plot was used as a single measure of model performance (Fielding & Bell 1997). For the threshold-dependent analysis, the results from ground validation surveys (see *2.4.4* below) of model predictions were used as test data in calculations of four measures of predictive success: sensitivity, specificity, correct classification, and Cohen's Kappa. Sensitivity and specificity are measures of the model's performance in correctly predicting presence and absence respectively. The correct classification rate is the proportion of correctly classified presences and absences according to model predictions (Barbosa *et al.* 2009). The Kappa statistic (Cohen 1960) defines the model's overall predictive accuracy on a scale of 0 (agreement with test data is no better than random) to 1 (perfect agreement).

2.4.4 Ground validation surveys

In order to ground validate threshold-dependent model predictions, a combination of acoustic monitoring and trapping methods were used to determine presence/absence of *B. barbastellus* within suitable (n = 13) and unsuitable (n = 17) woodlands throughout south-west Wales between June and September 2008 to 2009. For all woodland sites, surveys commenced 30 minutes before sunset and continued for 5 hours. Continuous passive acoustic monitoring of bat activity was performed using 3 Anabat bat detectors (Titley Scientific, Sydney, Australia) placed on woodland tracks and rides in locations where bats were expected to commute and/or forage. Trapping effort included one harp trap (2.4 x 1.85 m, Faunatech, Victoria, Australia) and 2 mist nets (2.6 x 6 m, Avinet, Dryden, NY, USA) used in conjunction with an acoustic lure (Sussex Autobat, Hill and Greenaway, University of Sussex, UK). The acoustic lure – programmed to emit a combination of *B. barbastellus* echolocation and social calls – has been shown to facilitate the capture of 'elusive species' (Hill & Greenaway 2005), hence its use was expected to minimise the effect of false negatives (failure to identify presence) on ground validation results. When caught, adult female bats were fitted with lightweight radio-transmitter tags (Pip3, 0.35g) (manufactured by Biotrack Ltd, UK) and radiotracked during the day using a Sika receiver (Biotrack Ltd., Wareham, UK) and a three-element hand-held Yagi antenna (Mariner Radar, Lowestoft, UK) to determine the location of roosts. Tags weighing <5 % of the body mass of the bat (Aldridge & Brigham 1988) were attached dorsally to the area between the scapulae using a form of biodegradable glue (Skin Bond, Pfizer Inc.). Tagging of pregnant and juvenile bats was avoided to minimise disturbance during these sensitive periods. When a roost was located, the specific roost cavity on each tree was identified using the directional antenna and by observation with binoculars from the ground. To determine if roosts were occupied by multiple bats (i.e. colony presence), emergence counts were conducted at dusk using Anabat bat detectors and by observation from the ground with binoculars and infrared video. To evaluate whether the methods used for ground validation surveys were adequate for identifying *B. barbastellus* presence (i.e. avoiding Type II (false negative) error), a subset of woodland sites were re-surveyed to see if the same presence/absence data was recorded on each occasion.

2.5 Results – Model 2

2.5.1 Analysis of model predictions

Of all the woodland in the study area, Maxent predicted relatively few areas as suitable for *B. barbastellus* (Fig. 2.6). The Jackknife analysis revealed that the environmental variable most important to the model was woodland type, followed by woodland area and density of broadleaved woodland (Fig. 2.4). Woodland type was also the variable that included the most information that was not present in other variables (i.e. the most uncorrelated information without which the model would lose most quality). Individual probability of presence plots for each of these variables show that *B. barbastellus* is most likely to occur in ancient broadleaved woodland (Fig 2.5a), in larger woodlands (Fig 2.5b), and in areas of high broadleaved woodland density (Fig 2.5c). The remaining environmental variables contributed comparatively little to the model (Fig. 2.4).

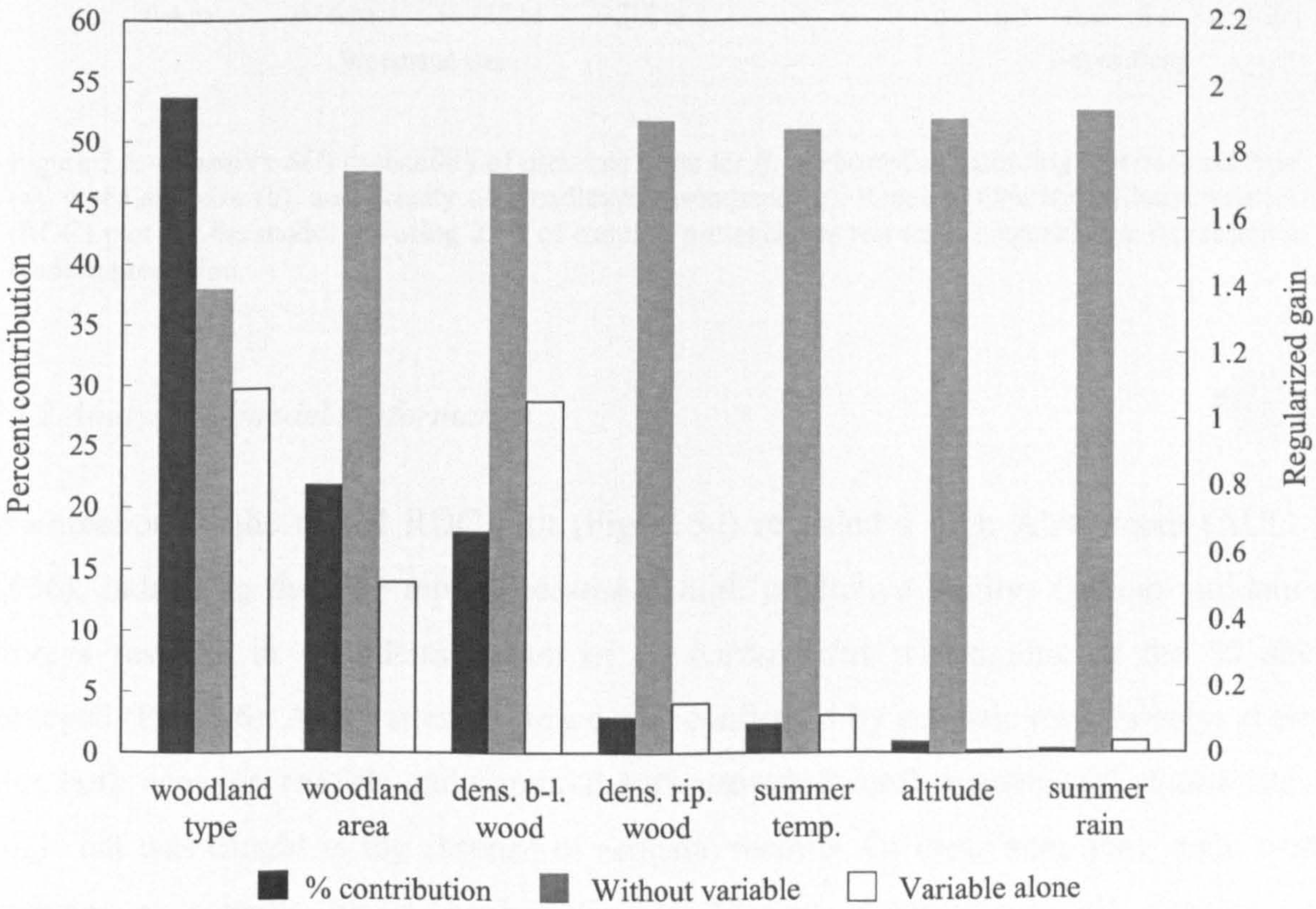

Figure 2.4 – Representation of the importance of each environmental variable to the model. Values for the percent contribution of variables (black bars) are scored on the left axis. Results of the Jackknife analysis are portrayed by grey bars (relative model performance when the corresponding variable is excluded) and white bars (relative model performance when only the corresponding variable is used) and are scored on the right axis. dens b-l wood: density of broadleaved woodland; dens rip wood: density of riparian woodland; summer temp: mean summer temperature. Refer to Table 2.2 for descriptions of environmental variables.

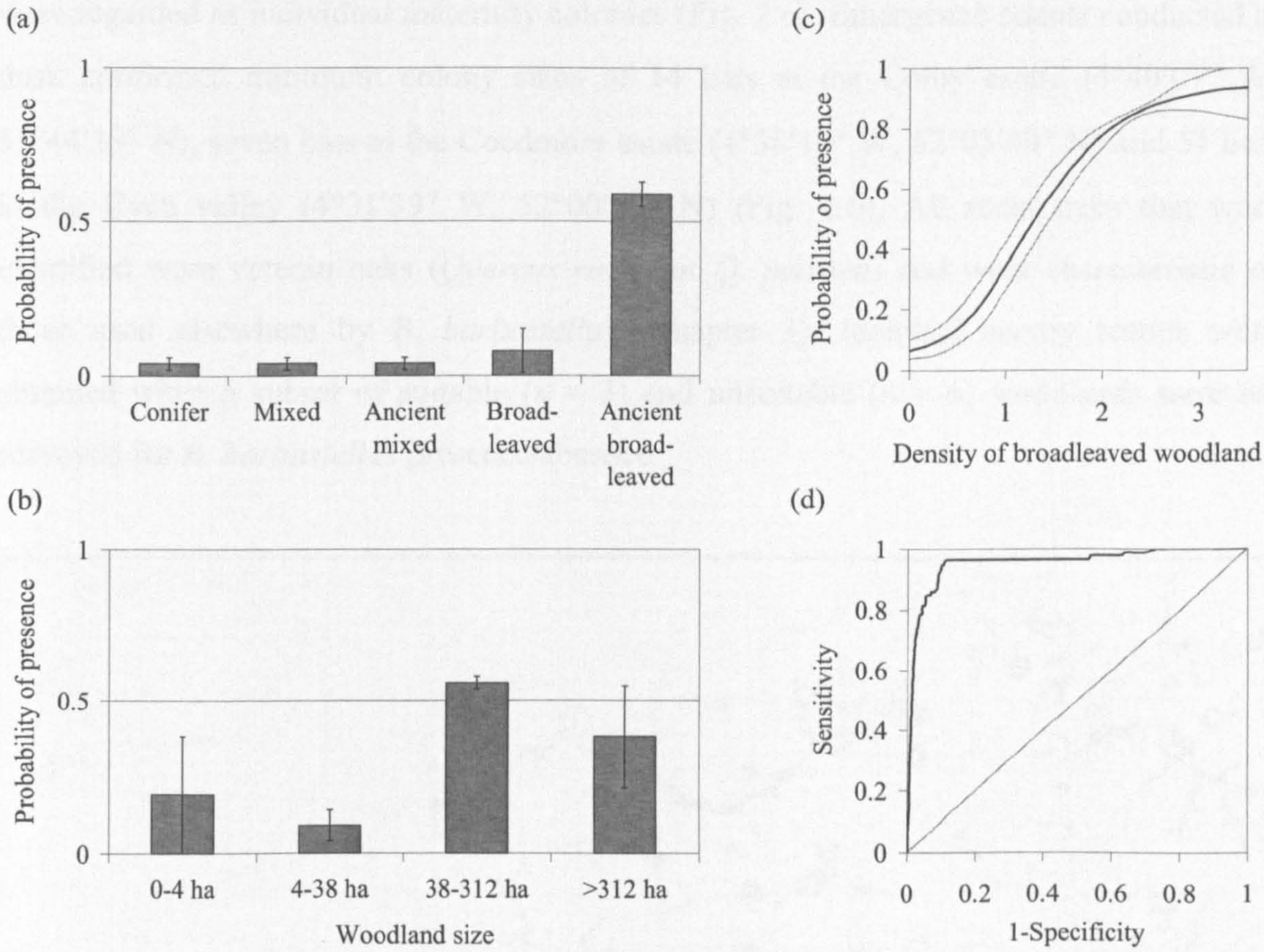

Figure 2.5 – Mean (± *SD*) probability of presence plots for *B. barbastellus* according to woodland type (a), woodland size (b), and density of broadleaved woodland (c). Receiver Operating Characteristics (ROC) plot for the model (d) using 25% of training presences as test data; diagonal line represents a random prediction.

2.5.2 Analysis of model performance

Examination of the model ROC plot (Fig. 2.5d) revealed a high AUC score (AUC = 0.956), indicating that the model possessed high predictive ability. Ground validation surveys resulted in the identification of *B. barbastellus* within nine of the 30 sites surveyed (Fig. 2.6). At six sites, presence was confirmed by acoustic records only; at two sites both acoustic records and captured bats provided confirmation; and at one site a single bat was caught in the absence of acoustic records. Of these nine sites, eight were predicted as suitable for *B. barbastellus* by Maxent. According to all measures of performance, the model performed with high predictive accuracy (sensitivity = 0.90; specificity = 0.80; correct classification rate = 0.83; Cohen's Kappa = 0.65). Radiotracking of caught bats resulted in the identification of three new maternity colonies. Because each one was separated by more than 7 km from any other, all three

were regarded as individual maternity colonies (Fig. 2.6). Emergence counts conducted at dusk confirmed minimum colony sizes of 14 bats at the Colby estate (4°40′07″ W, 51°44′29″ N), seven bats at the Coedmore estate (4°38′10″ W, 52°03′40″ N) and 51 bats in the Cych valley (4°31′39″ W, 52°00′21″ N) (Fig. 2.6). All roost trees that were identified were veteran oaks (*Quercus robur* or *Q. petraea*) and were characteristic of those used elsewhere by *B. barbastellus* (Chapter 3). Identical survey results were obtained when a subset of suitable (n = 3) and unsuitable (n = 6) woodlands were re-surveyed for *B. barbastellus* presence/absence.

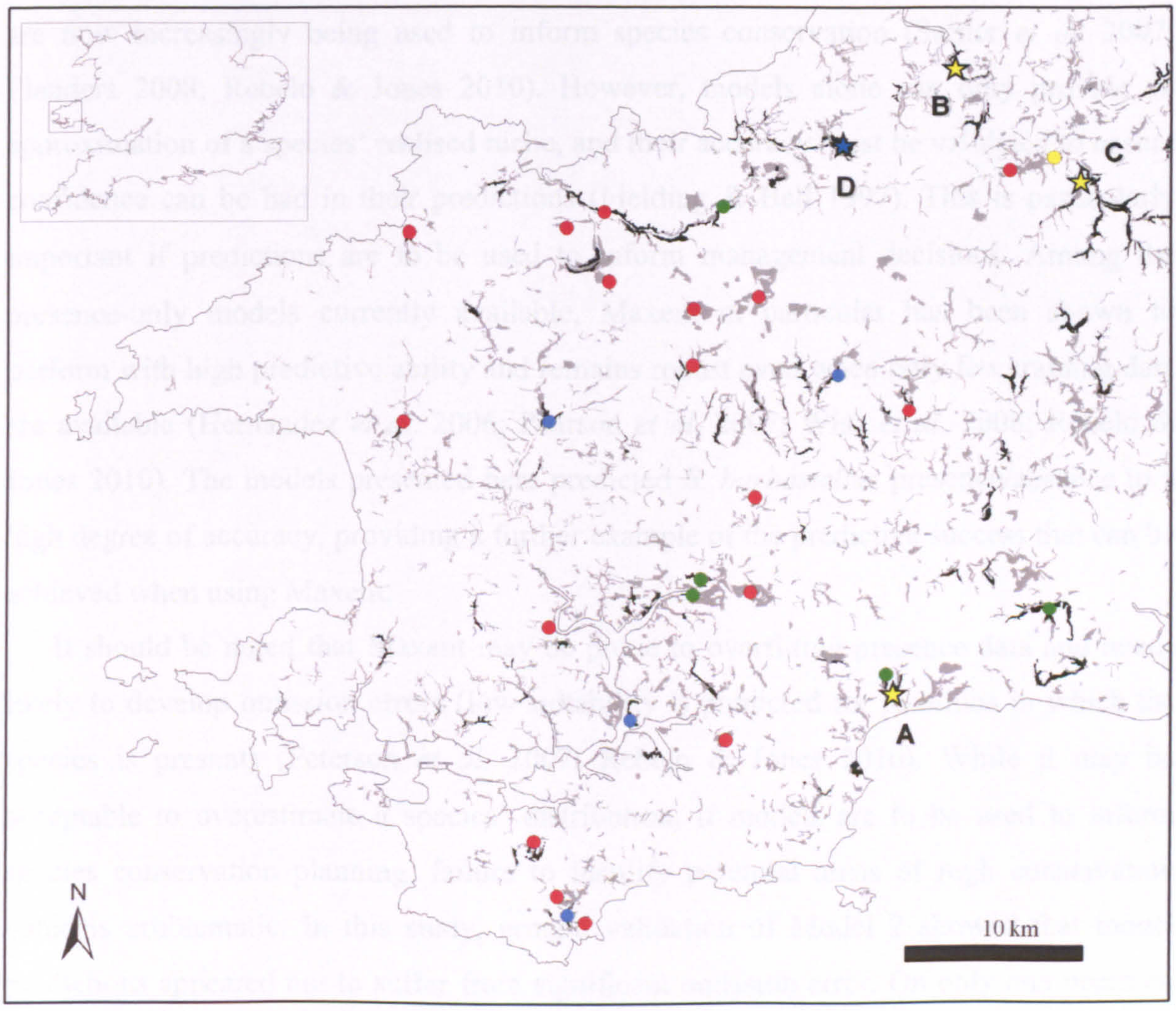

Figure 2.6 – Habitat suitability map for *B. barbastellus* in Pembrokeshire, south-west Wales, with results of ground validation surveys. Black areas represent woodlands in which *B. barbastellus* is predicted to occur and grey areas represent woodlands of predicted absence. Results of ground validation surveys are provided as true presence data (i.e. *B. barbastellus* presence was detected in a predicted presence site; green points) true absence data (i.e. no presence was detected in a predicted absence site; red points), false presence data (i.e. presence was detected in a predicted absence site; yellow points), and false absence data (i.e. no presence was detected in a predicted presence site; blue points). Locations of a previously recorded maternity colony (blue star) and newly discovered maternity colonies (yellow stars) are also displayed: Colby estate (A); Coedmore estate (B); Cych valley (C); and Pengelli Woods (D).

2.6 Discussion

2.6.1 Presence-only modelling of rare species

Presence-only modelling is proving to be an indispensable tool for identifying areas of high suitability for rare species (Papeş & Gaubert 2007; Flanders 2008), even when predictions are made outside of the species' current known range (Randin *et al.* 2006; Rebelo & Jones 2010). In addition, models can provide forecasts for how populations might be expected to respond to future environmental change (Guisan & Thuiller 2005; Beaumont *et al.* 2007; Rebelo *et al.* 2010; Winter 2009). Consequently, such techniques are now increasingly being used to inform species conservation (Sattler *et al.* 2007; Flanders 2008; Rebelo & Jones 2010). However, models alone can only provide an approximation of a species' realised niche, and their accuracy must be validated to ensure confidence can be had in their predictions (Fielding & Bell 1997). This is particularly important if predictions are to be used to inform management decisions. Among the presence-only models currently available, Maxent in particular has been shown to perform with high predictive ability and remains robust even when only few training data are available (Hernandez *et al.* 2006; Pearson *et al.* 2007; Wisz *et al.* 2008; Rebelo & Jones 2010). The models presented here predicted *B. barbastellus* presence/absence to a high degree of accuracy, providing a further example of the predictive success that can be achieved when using Maxent.

It should be noted that Maxent may be prone to overfitting presence data and hence likely to develop omission errors (low suitability is predicted for locations in which the species is present) (Peterson *et al.* 2007; Rebelo & Jones 2010). While it may be acceptable to overestimate a species' distribution, if models are to be used to inform species conservation planning, failure to identify potential areas of high conservation value is problematic. In this study, ground validation of Model 2 showed that model predictions appeared not to suffer from significant omission error. On only one occasion was *B. barbastellus* detected in an area of woodland considered unsuitable. Moreover, this presence was recorded in close proximity to an area of suitable woodland where two further presence records were made. Consequently, this record probably represents the detection of commuting bats from neighbouring areas of more suitable habitat.

To date, the majority of assessments of Maxent performance have focussed on predictive ability over large spatial scales (countries, continents), probably because the

more obvious applications of predictive models require modelling distributions across the full extent of a species' geographic range (e.g. forecasting responses to climate change (Beaumont *et al.* 2007)). Here, I show that Maxent also performs well when applied at higher spatial resolutions. Currently, however, modelling at this scale is somewhat limited by the availability of relevant habitat data. In this study, available datasets provided only a basic characterisation of woodlands and many features that are likely to be important in determining suitability (e.g. tree biometrics, canopy cover, and understorey composition) could not be modelled.

Among the variables that were used as environmental predictors for Model 2, those that described woodland structure were by far the most important to model performance. Evidently, *B. barbastellus* is highly dependent on large areas of native mature woodland, both for roosting (Greenaway 2001; Russo *et al.* 2004, 2005; Chapter 3) and for foraging (Sierro 1999; Chapter 4), and the availability of this habitat type is a significant factor limiting where the species can exist. Although the other environmental parameters measured were less informative, both summer temperature and summer precipitation – both of which are key parameters influencing offspring development and survival within maternity colonies (Racey 1973; Tuttle 1976) and the distribution of arthropod prey – were shown to be important predictors of *B. barbastellus* distribution at greater spatial scales (Model 1; see also Rebelo & Jones 2010). These variables were comparatively less informative in Model 2 probably because the climate throughout the modelled area (i.e. southern England and southern Wales) is relatively homogenous.

To date, there have been relatively few modelling studies of rare bat species, despite the obvious need to identify areas of high conservation importance to them. Reasons for this are thought to be associated with difficulties in detecting the species' presence in the field, such that either too few data are available to train models or model performance cannot be adequately tested via ground validation surveys (Engler *et al.* 2004). In this study, the repeated presence/absence records obtained from woodlands that were ground validated on more than one occasion suggest that a combination of acoustic monitoring and trapping with the aid of acoustic lures is an effective method for determining the presence or absence of *B. barbastellus* in the field.

The four maternity colonies now identified in south-west Wales represent the greatest concentration of *B. barbastellus* breeding sites currently known in the UK, indicating that a healthy population is resident there. Importantly, however, this population may be

isolated from the rest of the UK population by 100 km or more (Fig. 2.1). If this is indeed the case, this population may represent a unique gene pool that, by itself, should be a priority for conservation protection (Moritz 1994). A combination of genetic analysis and the expansion of ground validation surveys beyond areas of high suitability is advisable to evaluate the extent of genetic and spatial isolation and ultimately to determine whether specific measures should be taken to ensure population viability (Rebelo 2009).

2.6.2 Implications for conservation

Identifying the roost areas of maternity colonies is a key objective in bat conservation. This study has shown that habitat suitability modelling, when integrated with conventional field techniques, can be employed to great effect to facilitate the identification of maternity colonies among bats. Moreover, such an approach can significantly reduce the financial and time costs associated with a less systematic approach to surveying for rare species. In the future, increases in the availability of relevant habitat data, enhanced computer processing, and additional presence data will allow more complex models to be built that will make surveying for rare bat species ever more efficient.

Clearly, *B. barbastellus* is highly dependent on large areas of mature broadleaved woodland largely spared from forestry activities or intensive management (Greenaway 2001; Russo *et al.* 2004; Chapter 3). Importantly, within the study area much of this habitat was considered unsuitable because remaining woodland stands were either too small or too isolated. This is likely to be a problem inherent throughout much of the UK landscape. Consequently, to ensure the persistence of *B. barbastellus* in south-west Wales (and elsewhere in the UK), remaining areas of broadleaved woodland should be protected to prevent further fragmentation and should be managed to promote characteristics associated with more mature habitat – namely the presence of veteran and decaying trees, which typically provide more roosting opportunities for this species than do young trees (Chapter 3). In summary, the results of this study strongly support the use of predictive distribution modelling for conservation planning of rare bat species.

CHAPTER 3

Roost preferences and roosting behaviour

Abstract

Bats spend most of their lives in roosts. Moreover, roosts are important facilitators of sociality and reproductive success. As such, the roost preferences and roosting behaviour of rare bat species are a key consideration for their conservation. Here I investigate roost preferences and roosting behaviour of a breeding population of *Barbastella barbastellus* in south-west England. In total, 33 roost trees were identified by using radiotracking. Where possible, bats were located to roosts on consecutive days over extended periods to examine spatial and temporal patterns of roost switching behaviour. Trees in unmanaged semi-natural broadleaved woodland were favoured above those in other woodland types (plantation broadleaved woodland, plantation conifer woodland, forestry scrub) and roosts tended to be located close to rivers or steams that intersected woodland roosting areas. Preferences for roosting in dead oak (*Quercus robur*, *Q. petraea*) trees were shown, however, only the presence of one or more cavities on trees influenced significantly the selection of trees as roosts. Bats roosted in a variety of cavity types (defoliating bark, rot cavities, mechanical splits), however, no evidence of cavity selection was found based on the three characteristics measured (cavity type, height above ground, direction faced). Frequent roost switching by individuals and groups was observed suggesting that a large number of roosts are required by a single colony. Management of woodlands to promote characteristics associated with semi-natural broadleaved woodland, including large numbers of mature or dead trees, should be encouraged to provide roosting opportunities for *B. barbastellus*. Where possible, forestry activities within roosting areas should be avoided.

3.1 Introduction

Roosts are a vital commodity for bats. Fundamentally, roosts provide protection from predators and shelter from ambient environmental conditions, and are important sites for mating, hibernation and rearing young (Kunz & Lumsden 2003; Barclay & Kurta 2007). The availability of suitable roosts influences the distribution, diversity, social structure and reproductive fitness of bat communities; thus reductions in roosting opportunities can be highly detrimental to the persistence of local populations (reviewed in Kunz 1982; Kunz & Lumsden 2003). Echolocating bats utilise a variety of both ephemeral (e.g. leaves or defoliating bark) and more permanent (e.g. buildings or caves) structures for roosting, depending on their suitability and availability within different geographic and climatic zones (Kunz 1982). In temperate regions, many species roost predominantly in trees (e.g. Boonman 2000; Russo *et al.* 2004; Ruczyński & Bogdanowicz 2008). In particular, old or dead trees may be favoured presumably because they typically provide a greater abundance of suitable roost cavities (including defoliating bark, rot cavities, mechanical splits and woodpecker holes) than do healthy trees (Vonhof & Barclay 1996; Parsons *et al.* 2003; Arnett & Hayes 2009). The thermal properties and degree of exposure to predators associated with different cavity types are likely to be important factors influencing their specific selection by bats (Vonhof & Barclay 1997; Sedgeley & O'Donnell 1999a; Russo *et al.* 2004). Among colonial species the physical size of cavities is also likely to be important, dictating the number of bats using a single roost and so affecting their social organisation (Sedgeley & O'Donnell 1999a).

Many tree-dwelling bat species often utilise large numbers of roosts within a defined roost area (e.g. Boonman 2000; Russo *et al.* 2004; Willis and Brigham 2004). The rate at which bats switch between different roosts may vary considerably among species, or within species according to the availability of suitable roosts, the relative permanence of roost types, or the sex, reproductive state or seasonal activity of bats (Kunz 1982; Kunz & Lumsden 2003; Russo *et al.* 2005). Factors such as predation risk, parasite load within roosts, roost microclimate, social behaviour, and anthropogenic disturbance have all been hypothesised as being important for determining roost switching behaviour (Lewis 1995; Vonhof & Barclay 1996; Entwistle *et al.* 1997; Willis and Brigham 2004; Reckardt & Kerth 2007). While the individuals, or whole colonies, of some species exhibit roost switching on a daily basis, those of other species may continue to use a single roost for

extended periods of ten days or more (e.g. Brigham 1991; Kurta *et al.* 1993; Brigham *et al.* 1997; Russo *et al.* 2005). Depending on the extent of such behaviour, the number of available roosts within a defined roost area may place important constraints on the size and/or reproductive success of bat populations.

Loss of roosting opportunities associated with timber management and deforestation is considered a primary causal factor for the reduction in many European bat populations in recent history (Hutson *et al.* 2001; Racey & Entwistle 2003). Hence the accurate description of roosting requirements for bats is a key part of their conservation management. To date, few studies have characterised the roosting requirements of *Barbastella barbastellus.* Russo *et al.* (2004, 2005, 2010) documented roost preferences and temporal and spatial patterns of roost use in a breeding population from central Italy inhabiting extensive beech forests, however, only limited data are available for the species in the UK (Greenaway, 2001) where requirements may depart significantly from those of Italian populations. While records of summer roosts in buildings or in rock crevices have been documented (Harrington *et al.* 1995; Schober & Grimmberger 1997), radiotracking studies show *B. barbastellus* roosts predominantly in tree cavities and switches roosts frequently, often daily (Greenaway, 2001; Russo *et al.* 2004, 2005). Roosting areas are reused in consecutive years, although fidelity to specific roost trees may be less pronounced (Hillen *et al.* 2010). Given that bat roosts are afforded protection under UK and European law, such behaviour exhibited by tree-dwelling *B. barbastellus* has direct consequences for the management of woodland areas where breeding colonies exist.

Here I investigate roost preferences among a breeding population of *B. barbastellus* located within ancient woodland sites in south west England by examining selection on three levels (following Russo *et al.* 2004): woodland structure and management type; tree characteristics; and cavity characteristics. I test the hypotheses that selection is random on all levels by comparing the characteristics of roost features used by bats with those available in roost areas (Sedgeley & O'Donnell 1999b; Russo *et al.* 2004). I also present data on the spatial and temporal patterns of roost use and discuss all findings with regard to the implications for woodland management and the conservation of *B. barbastellus* in the UK.

3.2 Materials and methods

3.2.1 Study area

The study was conducted within Dartmoor National Park, in southwest England, between May and September, 2007 to 2009. *B. barbastellus* breeding colonies were identified by using radiotracking within three ancient woodland sites – Houndtor Wood (3°44′50″ W, 50°36′24″ N), White Wood (3°51′40″ W, 50°31′56″ N), and Dendles Wood (3°56′59″ W, 50°26′13″ N), hereafter referred to as Houndtor, White and Dendles – located within the upland valleys of the rivers Bovey, Dart and Yealm, respectively. The study area for each woodland was delimited, after locating roosts, according to woodland edges, ridges and other topographic features to encompass all roost plots. The study areas are separated by 10 to 12 km (see Fig. 4.1, Chapter 4) and share similar topographic features, including elevated (range 240-300 m a.s.l.) steep slopes of mainly granite substrate covered predominantly by oak (*Quercus robur*, *Q. petraea*) woodland. At Houndtor, previous forestry activities have delivered a mosaic woodland habitat containing a mix of semi-natural broadleaved (predominantly *Quercus* spp.), plantation broadleaved (mixed species), and plantation conifer woodland types (Fig. 3.1). Management policies aimed at restoring plantation stands to native woodland have generated areas of forestry scrub following limited and selective logging. All woodland sites are designated as Special Areas of Conservation (SAC).

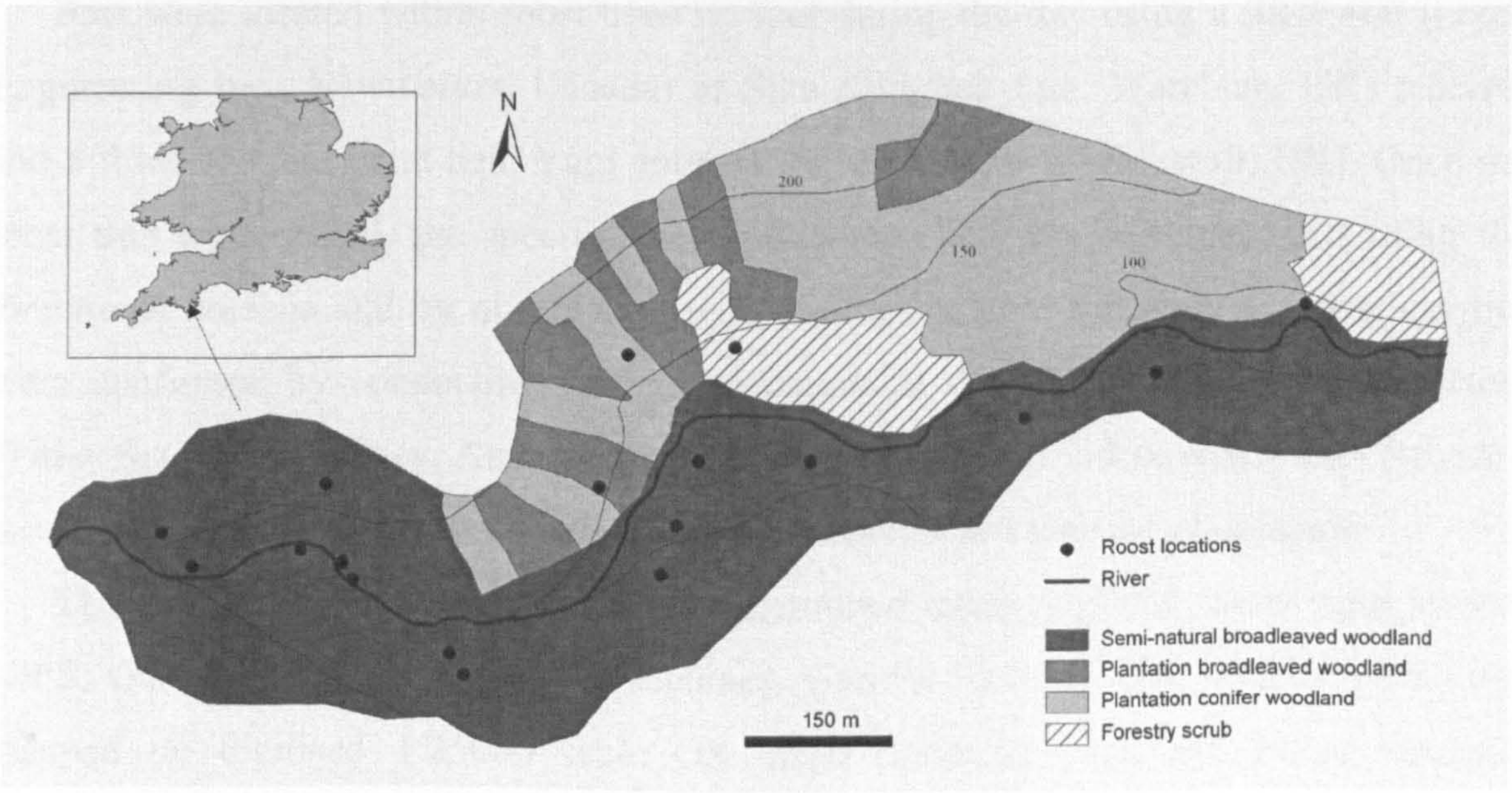

Figure 3.1 – Location of Dartmoor National Park (inset); map of Houndtor Wood study area including the location of 18 *B. barbastellus* roosts.

3.2.2 Bat capture and tagging

Bats were caught using mist nets and harp traps placed within woodlands and by using hand nets when bats emerged from known roost trees. An acoustic lure was used to improve catch efficiency in open woodland (Hill & Greenaway 2005). Following an assessment of condition (forearm length, weight, reproductive status), adult female bats were fitted with lightweight radio-transmitter tags (Pip3, 0.35g) (manufactured by Biotrack Ltd, UK) weighing <5% of the weight of the bat (Aldridge & Brigham 1988). After clipping away the fur, tags were attached dorsally between the scapulae using biodegradable glue (Skin Bond, Pfizer Inc.). Tagging of pregnant and juvenile bats was avoided on the grounds that additional stress caused by tagging procedures and from the burden of tags was considered potentially detrimental to the welfare of animals during these sensitive periods. All tagged bats were fitted with aluminium rings (3.5 mm, Mammal Society) to allow identification of recaptured individuals and prevent their repeated tagging. All activities were conducted under licence from Natural England.

3.2.3 Location of roosts and data recorded at roost sites

The methods detailed below adhere closely to those described by Russo *et al.* (2004, 2005) (following Sedgeley & O'Donnell 1999b) to allow direct comparisons *B. barbastellus* roosting behaviour to be made between studies.

Bats were located within roost trees on foot during the day using a SRX 400 (Lotek Engineering Inc., Newmarket, Canada) or Sika (Biotrack Ltd., Wareham, UK) receiver and a three-element hand-held Yagi antenna (Mariner Radar, Lowestoft, UK). Once the roost tree was located, the specific roost cavity on each tree was identified using the directional antenna and by observation with binoculars from the ground. Roost cavities were confirmed by conducting emergence counts at dusk using Anabat bat detectors (Titley Scientific, Sydney, Australia), binoculars and infrared video, which also provided an estimate of the number of bats that utilised the cavity and the size of colonies.

The location of each roost tree was determined using a global positioning system (GPS; Garmin eTrex H, 5- to 15-m accuracy, Garmin (Europe) Ltd., Romsey, UK) and mapped on digitised 1:25000 scale OS maps (Ordinance Survey; Edina Digimap Collections, www.edina.ac.uk/digimap/) and aerial photos (obtained from Natural England, Peterborough, UK) using ArcGIS 9.2 (Esri Inc., Redlands, CA, USA). The

terrain main aspect and percent slope of roost sites was measured using a compass and clinometer respectively. Elevation and distances from the nearest woodland edge, source of water and source of disturbance (i.e. tourist trails, roads or human habitation) were measured to the nearest metre in ArcGIS 9.2.

Roost and random (see 3.2.4 below) trees were classified as belonging to one of the following classes: Class 1 *Quercus*, i.e. live oak trees (*Q. robur* or *Q. petraea*) showing < 80% of dead limbs and loss of foliage; Class 2 *Quercus*, dead oak trees (80% or greater of dead limbs and loss of foliage); 'other broadleaved spp.' (all live individuals; includes beech, *Fagus sylvatica*; birch, *Betula pendula*; and maple, *Acer pseudoplatanus*); and 'conifer spp.' (all live individuals; includes Douglas fir, *Pseudotsuga menziesii*; and Scots pine, *Pinus sylvestris*). Features recorded from each roost tree included: height (measured with a clinometer), DBH (diameter at breast height), percent canopy closure (the degree of canopy closure around the tree assessed visually from the base of the tree), and total number of potential roost cavities visible with binoculars (9x magnification) from the ground on the trunk and main limbs. When roost cavities used by bats were identified, its type (defoliating bark, rot cavity, or mechanical split), height above ground (measured with a clinometer) and aspect (measured with a compass) were recorded.

Four quadrants around each roost tree were marked and the nearest tree potentially suitable for roosting in each quadrant was located (point-centred quarter method; Causton 1988). Each roost tree and its four neighbours constituted a roost plot (Sedgeley & O'Donnell 1999b). The retrospective approach to sampling within roost plots limited the selection of potential roost trees to those that were at least as high or as broad as the smallest roost trees. Hence, only those trees >3 m tall or with a DBH >17 cm were selected. The density of potential roost trees in each plot was calculated (in hectares) as 1000 / (mean of the four distances to nearest trees in metres)^2 and for each neighbour tree its type, height, DBH, percent canopy cover and number of cavities were recorded.

To investigate spatial and temporal patterns of roost use by *B. barbastellus*, tagged bats were located to day roosts on a daily basis to determine: (i) roost switching frequency, calculated as the number of switching events by each bat tracked continuously for at least three days divided by the number of tracking days, (ii) mean distance between consecutive roosts for each bat, (iii) mean straight distance travelled daily for roost switching by each bat. Only data for bats tracked continuously over at least three days were included in analyses.

3.2.4 Data recorded from random trees and cavities

To investigate whether *B. barbastellus* selected trees with particular characteristics for roosting, the features measured from roost trees were compared to those of 132 trees chosen at random. Random trees were located using the point-centred quarter method at 33 locations selected at random within study areas; each point-centre (a reference tree) with its set of four neighbour trees ('random' trees) constituted a random plot. Sampling effort was such that the number of random plots recorded in each of the three woodland sites was equal to the number of roost plots (Houndtor $n = 18$; White $n = 8$; Dendles $n = 7$). Random trees were chosen based on the same criteria used for selecting roost plot trees. From each random plot the same site and tree measurements were taken as were recorded at roost plots and for roost plot trees.

To investigate roost cavity selection, a total of 60 random cavities were located along transects within woodlands that intersected the areas in which most *B. barbastellus* roosts occurred. The identification of random cavities as potentially suitable roosts for bats was determined by eye using binoculars at ground level and based retrospectively on the structure of roost cavities used by *B. barbastellus*. In situations where trees supported multiple cavities, one cavity was selected at random. Within each woodland site two random cavities were recorded for each roost cavity that was identified. The same recordings were made from random cavities as were made from roost cavities (type, height above ground, aspect).

3.2.5 Statistical analysis

To determine habitat selection, a chi-square analysis was applied to examine whether the observed proportion of use (number of roosts occurring in each woodland category / total number of roosts located in the study area) departed from the expected proportion (calculated as the area of the corresponding woodland category / overall size of the study areas). Because study areas at White and Dendles contained only semi-natural broadleaved woodland, habitat selection was determined using only data from Houndtor. To meet chi-square analysis assumptions, woodland types (see 'study area') were lumped as 'semi-natural broadleaved' and 'other woodland' categories. Selection was established by calculating Bonferroni's confidence intervals for use of each woodland category (Neu

et al. 1974) using the standard normal deviate or Z statistic. ArcGIS 9.2 was used to map study areas and to measure the area occupied by each category.

The following univariate comparisons were carried out (following the methods of Russo *et al.* 2004):

- roost plots were compared with random plots to see whether there was any difference in elevation, terrain exposure, percent terrain slope, distances from nearest woodland edge, sources of water and disturbance;
- both roost trees and roost plot trees were compared with random trees to test for the occurrence of differences in tree type frequency, height, DBH, percent canopy closure, and total number of cavities;
- roost cavities were compared with random cavities to see whether their type frequency, height above ground and entrance direction differed significantly.

Because study areas shared similar topographical and habitat features, and because bats were recorded flying considerably greater distances (up to 20 km; see chapter 4) than the maximum distance between study areas (12 km), the data recorded from all study areas, for both tree and cavity characteristics, were pooled for statistical analyses. Mann-Whitney tests were used to compare samples where data sets were not normally distributed. Two-sample *t*-tests were used when distributions were normal. Frequency data were analysed with chi-square analyses. In chi-square tests, to avoid >20 % expected frequencies being <5 % (Dytham 1999), data from several categories were lumped together and Yate's correction was applied when necessary. Spearman's rank r_s coefficients were used to test for correlation between roost tree variables.

Logistic regression models were developed to determine which of the variables differing significantly between roost and random trees best explained any apparent selection. Logistic regression is particularly suitable for habitat association studies where habitat variables often have non normal distributions, are categorical, and the sampling design is retrospective (Ramsey *et al.* 1994; Sedgeley & O'Donnell 1999b). After initially fitting a full set of variables to the model, the significance of individual variables was tested by removing one variable at a time while leaving all others in place and measuring the corresponding reduction in deviance from the full model (Sedgeley & O'Donnell 1999b; Russo *et al.* 2004). Because logistic regression models presence and absence data,

the assumption was made that random trees were not used for roosting and were treated as absence data. Univariate tests were undertaken using Minitab release 16, and logistic regression was performed using SPSS release 11.5. In all tests, significance was set at $P < 0.05$.

3.3 Results

3.3.1 Bats radiotracked

A total of 19 adult female bats were radiotracked during May to September, 2007 to 2008; 14 were followed at Houndtor, four at White, and one at Dendles. Most bats (n = 18) were radiotracked during late July to September, after reproductive females had given birth and juveniles had become volant. Hence, the reproductive status of tracked bats was either post-lactating (n = 11) or nulliparous, i.e. they had not given birth in the year of radiotracking (n = 9). Juvenile bats were caught during the study period but were not tagged. Tagged bats were radiotracked for as long as transmitters remained attached or functional ($\bar{X}$ = 14.1 days ± 3.3 *SD*, range 8-20 days), resulting in the identification of 28 roost trees. A further five roost trees that were previously identified at Dendles between 2000 and 2003 (Geoff Billington, unpubl. data) were also included in the dataset.

3.3.2 Landscape characteristics of roost sites

Tagged bats used roost trees located in all woodland types: 30 roosts were found in semi-natural broadleaved woodland and one in each of plantation broadleaved, plantation conifer and forestry scrub woodland categories. Chi-square analysis showed that roost trees were not distributed at random across woodland categories; semi-natural broadleaved woodland was positively selected, while 'other woodland categories' were used in proportion to their availability (Table 3.1). Roost sites were located closer to water ($\bar{X}$ = 36 m ± 26 *SD*; range 1-93 m) than random sites (t = –4.72, df = 49, $P < 0.001$), however, comparisons for all other characteristics – altitude, slope gradient, terrain main aspect, and density of potential roost trees – showed no significant difference (chi-square, Man-Whitney, and t-tests not significant).

Table 3.1 – Results of chi-square and selection analyses (Bonferroni's confidence intervals) for woodland categories according to habitat type. Proportion of use expected = area of 'woodland type'/overall size of the study area; Proportion of use observed = number of roosts occurring in the corresponding woodland type/number of roosts in the study area.

Woodland type	Area (ha)	Number of roosts	Proportion of use expected	Proportion of use observed	Chi-square value	Selection
Semi-natural broadleaved	24.0	15	0.522	0.833	2.773	Positive
Other woodland categories[†]	22.0	3	0.478	0.167	3.028	Absent
Total	46.0	18	1	1	5.801*	

[†] Includes plantation broadleaved woodland, plantation coniferous woodland, and forestry scrub. * $P < 0.05$

3.3.3 Selection of roost trees

The majority of roost trees (17 out of 33) were Class 2 (dead) *Quercus* trees. Of the remaining 16 roost trees, 15 were Class 1 (alive) *Quercus*, and one was a live beech tree (*Fagus sylvatica*). Conifer species were not used as roosts. Comparatively, random trees totalled 70 Class 1 *Quercus* (53%), 13 Class 2 *Quercus* (10%), 31 'other broadleaved' (23%), and 18 conifer (14%). To meet chi-square analysis assumptions, 'conifer' was removed from the dataset. Chi-square analysis showed that tree types were not selected at random by roosting bats; Class 2 *Quercus* was positively selected, Class 1 *Quercus* was used in line with availability, and other broadleaved species were used less than expected (Table 3.2).

Table 3.2 – Results of chi-square and selection analyses (Bonferroni's confidence intervals) for tree classes according to tree species and condition (class 1: alive; class 2: dead; other broadleaved = all live trees). Proportion of use expected = number of random trees in each tree class/total number of random trees; Proportion of use observed = number of roost trees in each tree class/total number of roost trees.

Tree class	Roost trees	Random trees	Proportion of use expected	Proportion of use observed	Chi-square value	Selection
Class 1 *Quercus*[†]	15	70	0.614	0.455	1.119	Absent
Class 2 *Quercus*[†]	17	13	0.114	0.515	43.130	Positive
Other broadleaved[‡]	1	31	0.272	0.030	6.227	Negative
Total	33	114	1	1	50.476***	

[†] Includes *Q. robur* and *Q. petraea*. [‡] Includes beech, *Fagus sylvatica*; birch, *Betula pendula*; and maple, *Acer pseudoplatanus*. *** $P < 0.001$

Overall, roost trees (n = 33) were shorter (t = –3.03, df = 43, P < 0.01) and had more cavities ($W_{33, 132}$ = 4525.5, P < 0.001) than random trees (n = 132); no difference was recorded for DBH or percent canopy closure (Fig. 3.2). When tree classes were analysed separately, Class 1 *Quercus* trees that were used for roosting (n = 15) supported higher numbers of cavities than did random trees of the same class (n = 70) ($W_{15, 70}$ = 1078.5, P < 0.001); all other comparisons were not significant (Fig. 3.2). When roost plot trees were compared with random trees, no differences were found for any of the characteristics measured (Fig. 3.2).

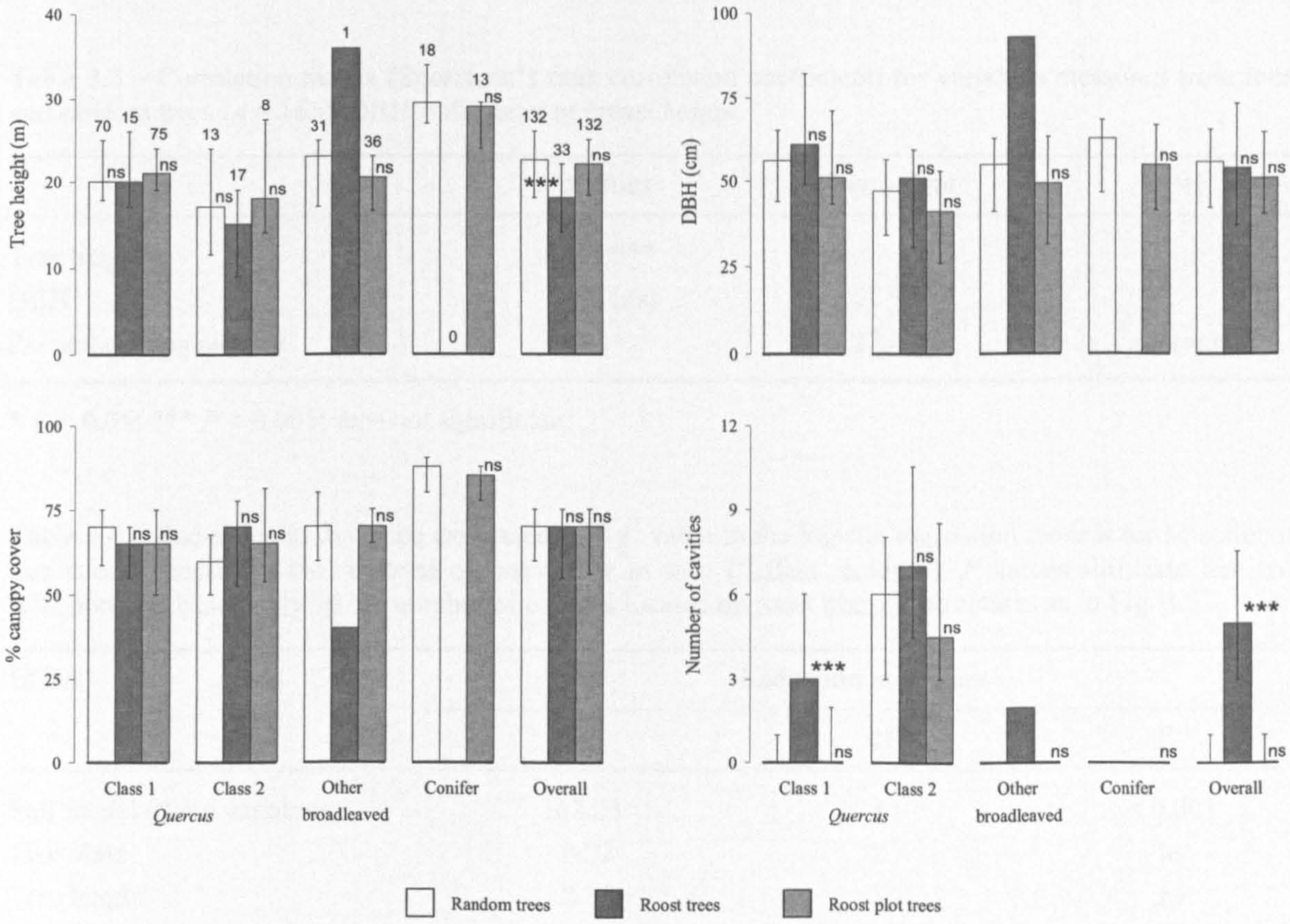

Figure 3.2 – Median and interquartile range of tree height, DBH (diameter at breast height), percent canopy closure and number of cavities for random trees, roost trees and roost plot trees, and results of Mann-Whitney and t-tests (features of roost and roost plot trees were compared with those of random trees both within each tree class and in the overall sample). Class 1 *Quercus* = live oak (including *Q. robur* and *Q. petraea*) trees (<80% of dead limbs and loss of foliage); Class 2 *Quercus* = dead oak (>80% of dead limbs and loss of foliage); 'other broadleaved' (including beech, *Fagus sylvatica*; birch, *Betula pendula*; and maple, *Acer pseudoplatanus*) and conifer (including fir, *Pseudotsuga menziesii*; and pine, *Pinus sylvestris*) = all live trees. No analysis was performed for roost versus random trees of the classes 'other broadleaved' and conifer since only one roost tree was classified as 'other broadleaved' and no roosts were found on conifer trees. Numbers labelling bars in 'tree height' graphs indicate samples sizes. *** P < 0.001; ns = difference not significant.

Most of the variables considered for tree characteristics were significantly correlated (Table 3.3). To identify which of the variables that differed significantly between roost and random trees – tree type, tree height, number of cavities – actually influenced selection, a logistic regression model was derived using these variables. The full model was significant ($\chi^2 = 62.23$, df = 4, $P < 0.001$) and a goodness-of-fit test did not reject the null hypothesis of an adequate fit (Hosmer and Lemeshow test: $\chi^2 = 5.79$, df = 8, $P < 0.67$). The model classified correctly 81.6% of available (random) trees and 78.8% of used (roost) trees (overall correct classification = 81.0%, $n = 147$). The full model was only affected significantly when 'number of cavities' was removed (Table 3.4).

Table 3.3 – Correlation matrix (Spearman's rank correlation coefficient) for variables measured from roost and random trees ($n = 165$). DBH = diameter at breast height.

	n cavities	Tree height	DBH
Tree height	– .24 ***		
DBH	– .08 (ns)	.61 ***	
Percent canopy closure	– .19 *	.27 ***	.06 (ns)

* $P < 0.05$; *** $P < 0.001$; ns = not significant.

Table 3.4 – Reduction in deviance expressed as a χ^2 value in the logistic regression models for selection of tree roosts caused by the removal of one factor in turn ('Effect' column). *P* values illustrate that tree selection was based only on the number of cavities located on roost trees. Tree classes as in Fig. 3.2.

Effect	Reduction in deviance		
	χ^2	df	*P*
Full model (three variables)	62.23	4	< 0.001
Tree class	0.22	2	ns
Tree height	2.89	1	ns
Number of cavities	34.50	1	< 0.001

ns = not significant

3.3.4 Selection of roost cavities

Of the 33 trees used for roosting, the specific roost cavity used by bats was identified in 30 cases. Bats roosted most frequently under defoliating bark ($n = 17$), followed by rot cavities ($n = 8$) and mechanical splits ($n = 5$). Comparatively, random cavities recorded from 60 trees totalled 30 cavities under defoliating bark (50%), 21 rot cavities (35%) and

9 splits (15%). Chi-square analysis revealed no difference in the relative frequency of cavity types used for roosting with that of available cavities. On average (± *SD*), the height at which roost cavities were located above ground level was 5.2 ± 2.7 m, and roost cavities faced north (n = 13) or south (n = 17) with similar frequency. As was found for cavity type, neither the height of roost cavities nor the distribution of directions of cavities differed from that of random cavities (t-test and chi square analysis not significant).

3.3.5 Temporal and spatial patterns of roost use

Patterns of roost use were determined for 18 individual *B. barbastellus*. Bats were located within roost trees on consecutive days for periods of 11.4 days ± 4.0 *SD* (range 5-18 days). All bats switched roosts on a regular basis with individuals occupying roosts, either independently or most often with other colony members, for just 2.3 days ± 2.0 *SD* (range 1-5 days) before moving to an alternative roost. Emergence counts revealed roosts held 10.8 bats ± 7.3 *SD* (range 1-23 bats; n = 23 roosts). When bats switched roosts, the average distance between consecutive roost trees was 288 m ± 121 *SD* (range 180-711 m; n = 18 bats). Typically, individual bats travelled 126 m ± 87 *SD* each day (n = 18 bats) to accommodate roost switching behaviour. When data for post-lactating (n = 10) and nulliparous (n = 8) bats were compared, no difference was found in either the distance between consecutive roosts ($W_{10, 8}$ = 109.5, P = 0.21) or the frequency of roost switching ($W_{10, 8}$ = 112.0, P = 0.14). Data for roost switching behaviour are summarised in Table 3.5.

Table 3.5 – Mean distance moved between consecutive roosts (calculated from the means of individual bats) and roost switching frequencies (number of switches/number of radiotracking days) calculated for bats tracked continuously over at least three days. n = number of bats; *SD* = standard deviation. No difference was found between post-lactating and nulliparous for either variable (Mann-Whitney tests).

Status		Mean distance moved (m)		Roost switching frequency	
	n	Mean (*SD*)	Range	Mean (*SD*)	Range
Post-lactation	10	323 (152)	192-711	0.47 (0.09)	0.33-0.60
Nulliparous	8	245 (44)	180-313	0.41 (0.06)	0.33-0.50
W		109.5		112.0	
P		0.21		0.14	
All bats	18	288 (121)	180-711	0.44 (0.08)	0.33-0.60

3.4 Discussion

3.4.1 Roost preferences and roosting behaviour

Selection of roosts by *B. barbastellus* in this study was not random. Bats made significant use of dead oak (*Quercus robur* and *Q. petraea*) trees, confirming the importance of dead and decaying trees to this species for roosting (Greenaway 2001; Steinhauser *et al.* 2002; Russo *et al.* 2004). Although rare throughout all three study areas, at Houndtor, dead trees occurred most frequently in unmanaged semi-natural broadleaved woodland, where the number of roosts identified was higher than expected from the availability of this woodland type. The extent of decay or damage on most dead roost trees was such that their height was well below that of the surrounding canopy; hence, overall, roost trees were shorter than the surrounding stand. In contrast, many tree-roosting species, including *B. barbastellus* in central Italy (Russo *et al.* 2004), have been found to select trees that are taller than the surrounding stand, presumably because larger trees that project above the canopy are easier to locate and may offer warmer roost microclimates due to greater exposure to solar radiation (Vonhof & Barclay 1996; Brigham *et al.* 1997; Sedgeley & O'Donnell 1999b).

Given that the summer climate of roost areas in this study is cooler and wetter than that of central Italy, the selection by *B. barbastellus* in this study of short trees with limited solar exposure was unexpected and challenges the assumption that in cool-temperate environments tree-roosting bats will select roosts to optimise solar exposure. The difference in roost tree selection by bats in this study and that of Russo *et al.* (2004) may be explained by differences in the reproductive state of bats; Russo *et al.* (2004) studied only breeding females while in this study only non-breeding females were studied. Warmer roosts may be most beneficial to breeding females, allowing them to maintain homeothermy during pregnancy and lactation, therefore facilitating foetal development and accelerating juvenile growth (Racey 1973; Tuttle 1976; Racey & Swift 1981; Hoying & Kunz 1998). Conversely, non-breeding females may conserve energy during the day most efficiently by becoming torpid in cooler roosts (Kerth *et al.* 2001a). This might also explain the selection of high, south facing cavities by *B. barbastellus* in Italy (Russo *et al.* 2004) and the random selection of cavities in my study.

Despite the apparent preference for short dead oak trees, the results of the logistic regression analysis showed that only the number of suitable cavities on trees was

important in describing selection of roost trees. Given that most random trees displayed no suitable cavities, roost tree selection may depend simply on the availability of just one cavity rather than an abundance of cavities *per se*. This has important implications for woodland management as any tree that supports just one suitable roost feature, independent of tree species or condition, may be used for roosting by bats and hence may need to be afforded protection. Although tree class did not influence selection, dead trees are likely to be of high value because typically they contribute more suitable roosting opportunities than do live trees and may offer more preferred thermal conditions. In this study, even when live trees were used as roosts, roost cavities were commonly located on dead or decaying limbs. Oak trees may also be especially valuable because typically they remain standing after death and retain dead limbs. Furthermore, because they senesce very slowly, oak trees may continue to develop new roosting opportunities over many years, thereby reducing the need for bats to search for new roost locations.

Of all the characteristics measured at roost locations, only distance to water appeared to influence selection. Within each study area the respective river that flowed through it was a prominent feature of the landscape and the nearest source of water to all roosts (see Fig. 3.1 for example). Thus, waterways that intersect roosting areas may be important spatial and perhaps also acoustic cues used by bats to locate roosts. The need to drink and the importance of riparian zones for foraging (Chapter 4) may also influence the selection of roosts close to water. Although a dense understorey has previously been considered to be an important component of the roosting environment for *B. barbastellus* (Greenaway 2001), in this study significant understorey was rare. Therefore, while in some circumstances bats may capitalise on warmer microclimate conditions associated with the presence of dense understorey, it appears not to be a prerequisite of a 'suitable' roost.

Roost switching appears to be a common behaviour of *B. barbastellus*, as is true of other tree-dwelling bat species (Lewis 1995; Kerth & König 1999; Kunz & Lumsden 2003; Willis & Brigham 2004). The extent of this behaviour expressed by bats in this study as well as estimated colony sizes was similar to that recorded for the species elsewhere (Russo *et al.* 2005). Although its specific purpose is unknown, roost switching is likely to be influenced by numerous factors, including predation risk, parasite load within roosts, roost microclimate, social behaviour, and may also be important in reinforcing knowledge of multiple roost locations, providing bats with readily accessible roosting opportunities should an occupied roost become unfavourable.

3.4.2 Implications for conservation

The availability of mature or dead broadleaved trees is a critical component of the roosting environment for *B. barbastellus*. Moreover, the relatively small colony sizes and frequent roosting switching behaviour exhibited by this species implies that even a relatively few individuals may require a considerable number of such features within a defined roost area (Russo *et al.* 2004, 2005). Hence, woodlands identified as roosting areas should be managed to promote the preservation and development of mature and dead trees as well as trees supporting prominent dead limbs, even when present as remnants within plantation conifer stands. More generally, characteristics associated with ancient broadleaved woodland should be encouraged throughout roosting areas, but particularly along or adjacent to rivers or streams, or other water bodies, when present. According to Russo *et al.* (2004) *B. barbastellus* is probably unable to find suitable roosting sites where intensive and non-selective logging is conducted. Consequently, felling operations should be avoided as far as possible within known roosting areas and should affect only isolated areas within any one year. When such activities are unavoidable, surveys for trees supporting potentially suitable roost cavities should be undertaken prior to the commencement of logging and involve careful inspection of identified cavities for the presence of bats.

Bats in this study were never recorded moving away from roosts when approached by trackers during the day, as has occasionally been recorded elsewhere (Russo et al. 2004). Indeed, some roosts were located directly adjacent to footpaths frequently used by tourists. Despite this, in agreement with Russo *et al.* (2004) tracks and paths, where created to facilitate logging operations or recreational activities, should avoid likely *B. barbastellus* roosting areas and known roost locations to minimise disturbance.

Lastly, because woodland adjacent to that within which breeding colonies are located may support sub-colonies (Greenaway 2008), management efforts directed at conserving sufficient roosting habitat for *B. barbastellus* should incorporate areas of woodland of a greater extent than that outlined by known tree roosts alone. Given that tree-dwelling bats typically show high seasonal and long-term fidelity to roosting areas (Lučan *et al.* 2009; Hillen *et al.* 2010), focussed management practices that deliver the roosting needs of *B. barbastellus* are likely to have long-term benefits for the species.

CHAPTER 4

Home range use and habitat selection

Abstract

Anthropogenic modification of natural habitats has resulted in widespread population declines among many bat species. Loss of foraging habitat and associated reductions in insect prey are considered central to this problem. The identification and protection of habitats most important to foraging bats is therefore a key part of their conservation management. Here I investigate home range use, habitat selection and foraging behaviour in a breeding population of *Barbastella barbastellus* in southwest England. In total, 19 adult female bats were radiotracked to determine home range sizes and to identify core foraging areas. Habitat selection was examined using compositional analyses. Individual home ranges varied considerably, with bats travelling between one and 20 km to reach foraging areas ($\bar{X}$ = 8.4 km ± 4.9 *SD*). Females were highly faithful to more or less 'private' foraging areas which constituted a small fraction ($\bar{X}$ = 10.1 % ± 8.8 *SD*) of home ranges. Riparian vegetation and broad-leaved woodland were the habitats most strongly selected for foraging. Unimproved grassland and field margins were also important components of the foraging environment. Urban, arable and upland moor habitats were least selected. Conservation management policies for *B. barbastellus* should target the protection and enhancement of key foraging habitats primarily within 8 km of roost sites. Linear landscape elements such as tree lines and hedgerows should also be managed to improve their value to foraging bats.

4.1 Introduction

The availability of productive foraging habitat is a key factor influencing the distribution and survival of species. For species that follow extreme 'K-strategy' life histories such as bats, the loss or reduction in quality of foraging habitat and its fragmentation may significantly undermine the reproductive output and long-term persistence of populations (Racey & Entwistle 2003). Throughout the past century, anthropogenic modification of natural landscapes has greatly altered the structure and functionality of ecosystems (Tilman *et al.* 2001; Robinson & Sutherland 2002; Foley *et al.* 2005). In Europe, loss of foraging habitat associated with urbanisation and agricultural intensification, and further reductions in prey associated with pesticide use are considered perhaps the most significant contributory factors to population declines among many bat species (Stebbings 1988; Hutson *et al.* 2001). Future effects of climate change on the structure and dynamics of bat and insect communities are likely to further exacerbate the threat to these ecologically important predators (McCarty 2001; McLaughlin *et al.* 2002; Rebelo *et al.* 2010). As the pressure on ecosystems from human activities continues to mount, accurate descriptions of the exacting ecological requirements of individual taxa are vital for identifying those species most at risk and for developing effective management strategies to protect the most vulnerable.

Although habitat preferences have been described for numerous bat species (e.g. Walsh & Harris 1996; Russ & Montgomery 2002), for many others there remains a significant lack of quantitative data on the relative importance of different habitat types for foraging. Currently, very little is known regarding the foraging behaviour and habitat requirements of *Barbastella barbastellus*. The species' rarity, its low amplitude echolocation (Goerlitz *et al.* 2010), and its use of roosts primarily in woodland (Russo *et al.* 2004; see also Chapter 3) make its detection in the field extremely difficult, and probably account for why it remains largely understudied and little known. Recent autecological studies describe large home ranges, distinct spatial organisation of foraging sites among colony members, and high foraging site fidelity (Hillen *et al.* 2009). Sierro (1999) investigated habitat selection by a small population of *B. barbastellus* in the Swiss Alps, where radiotracked bats showed a clear preference for richly structured forests with high biological productivity and an avoidance of open woodland on stony outcrops and rocky slopes. Such upland landscapes however are not typical of those found within much

of this species' Europe-wide range and habitat preferences within lower lying agricultural landscapes, which dominate land cover throughout Europe, may be quite different.

In this study I use radiotracking and compositional analysis to investigate ranging behaviour and habitat selection in a breeding population of *B. barbastellus* in southwest England. I test the hypotheses that (a) individual bats forage spatially at random within home ranges and (b) foraging bats use habitat types in proportion to their availability. Qualitative observations on the spatial organisation of foraging sites, foraging site fidelity, and patterns of nocturnal activity are also discussed. *B. barbastellus* has a highly specialised diet comprising almost entirely moths (Chapter 6). It is therefore predicted that habitat types supporting high seasonal abundances of moths will be selected preferentially by this species. Ultimately, this study aims to address key objectives within the *B. barbastellus* Species Action Plan (SAP; Anon 1998) and contribute to the development of a conservation plan for the species.

4.2 Materials and methods

4.2.1 Study areas and habitat mapping

Three study areas were delimited, after analysis of radiotracking data, according to the extent of home range areas around each of three *B. barbastellus* maternity colony roost sites (Fig. 4.1). For a description of the woodland roost sites – Houndtor, White, and Dendles – refer to *3.2.1*. The study areas are separated by less than 10 km and located within a heavily undulated landscape on the periphery of Dartmoor National Park, a 954 km^2 area characterised by elevated moors and granite outcrops. Pastoral farmland dominates the hillsides and lower landscape while dense woodland occurs as scattered blocks throughout and is retained on many steeper slopes. Localised, often intensive areas of urban and arable land use are also notable features of the lower landscape.

Habitat data for study areas were extracted from aerial photographs (supplied by Natural England, Peterborough, UK) and from the databases of the Dartmoor National Park Authority and Devon Biodiversity Records Centre. These data were validated through ground surveys undertaken at the time of radiotracking and found to be accurate. Land-use maps were created for study areas within ArcGIS 9.2 (Esri Inc., Redlands, CA, USA) using the 11 broad habitat categories described in Table 4.1.

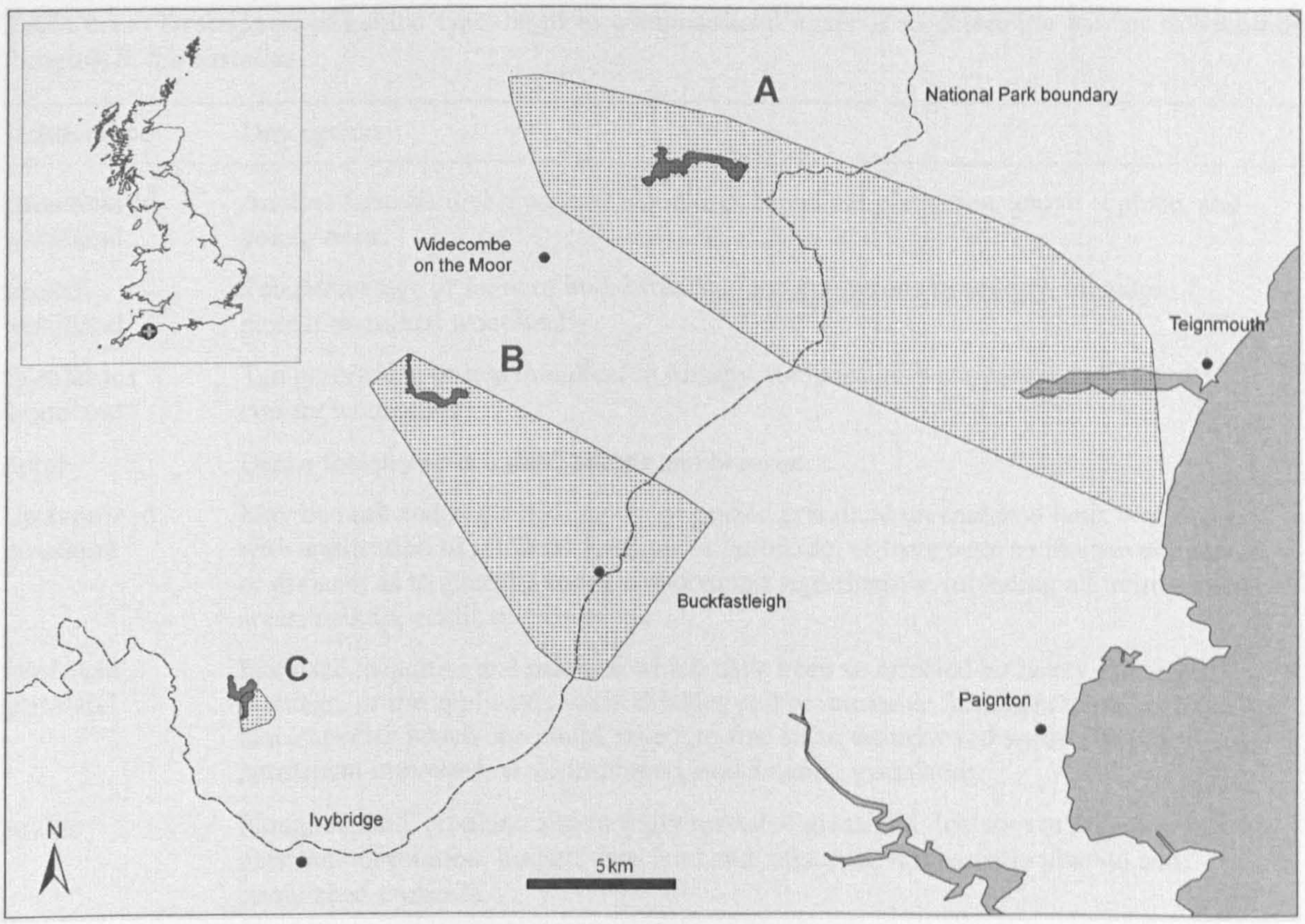

Figure 4.1 – Map of study area, south-east Devon, England; shows the combined home range areas (dotted polygons) of adult female *B. barbastellus* radiotracked from three woodland maternity roost areas (solid grey polygons) at A: Houndtor (n = 14 bats); B: White (n = 4 bats); C: Dendles (n = 1 bat). Combined home range areas were calculated as 100% minimum convex polygons delimiting all radiotracking fixes for all bats within each study area.

4.2.2 Bat capture and radiotracking

For details of the methods by which bats were captured and tagged, refer to *3.2.2*. Tagged bats were followed using a Sika receiver (Biotrack Ltd, Wareham, UK) and a 3-element Yagi antenna, and their locations recorded every 5-10 minutes from dusk to dawn (Jones & Morton 1992; Duvergé 1996) using the 'homing-in' method (White & Garrott 1990). For each data point, tracker location was recorded to a ten figure grid reference using a global positioning system (GPS) (Garmin eTrex H, 5- to 15-m accuracy, Garmin (Europe) Ltd., Romsey, UK) and the direction of peak signal was recorded using a compass. The distance from tracker to bat was estimated using receiver gain and signal strength. Any night of data resulting from less than 95% contact time with a bat was excluded from final analyses as the complete pattern of movements throughout the night could not be identified.

Table 4.1 – Description of habitat types used in compositional analysis to determine habitat selection by foraging *B. barbastellus*.

Habitat type	Description
Broadleaved woodland	Ancient semi-natural broadleaf woodland, broadleaf plantation, active coppice, and young trees.
Mixed woodland	Ten percentage or more of both broadleaf and coniferous in canopy, includes plantation mixed woodland.
Coniferous woodland	Ten percentage or less broadleaf in canopy, includes both plantation and natural conifer woodlands.
Scrub	Dense forestry scrub, small shrubs and bracken.
Unimproved grassland	May be rank and neglected, mown or grazed grassland on enclosed land. Not treated with application of artificial fertiliser or herbicide, or have been so intensively grazed or drained, as to alter the sward composition significantly, including all unimproved areas, neutral, acidic or calcareous.
Improved grassland	Enclosed meadows and pastures which have been so affected by heavy grazing, drainage, or the application of herbicides and/or inorganic fertilisers that they have lost many species which one could expect to find in an unimproved sward. Includes permanent improved, semi-improved, and amenity grasslands.
Arable	Ploughed land, cropland and recently reseeded grassland. Includes arable land and grassland in rotation, horticultural land and nurseries, and recently planted and established orchards.
Riparian	Marginal vegetation around any water body, including riparian woodland, tall vegetation along water courses, swamp vegetation around pools and all types of fen and mire.
Open water	Open water, including rivers, streams, brooks, lakes, ponds (including operational ponds), reservoirs, aquaculture, and estuary and coastal waters.
Urban	Roads, houses and residential land, built-up areas, including areas of commercial retail, industry, high density residential (>40% cover), agricultural buildings, transport areas, restored or active landfill sites, and active or inactive quarries.
Upland moor	Unenclosed areas of unimproved upland habitat, often grazed, including wet and dry shrub heath, heath grassland mosaic, gorse, bracken and acid grassland.

Hedgerows and minor tree lines, where present as secondary habitats, were included in grassland, arable, riparian and urban categories.

4.2.3 Analysis of ranges and habitat preference

Radiotracking fixes for each individual bat were mapped on digitised 1:25000 scale OS maps (Ordinance Survey; Edina Digimap Collections) and aerial photos (obtained from Natural England, Peterborough, UK) using ArcGIS 9.2. Digitised radiotracking data were analyzed in Ranges 7 (Anatrack Ltd, Wareham, UK) to determine home ranges. 100% minimum convex polygons (MCPs) were used to define both individual home ranges (delimiting all fixes corresponding to each bat) and combined home ranges (delimiting all fixes from all colony members). Cluster polygons were considered the most appropriate

minimum-linkage estimators to define the core areas in which bats foraged as locations collected from each individual could not be assumed as sufficiently independent for location density estimators of home range that make parametric assumptions (Kenward 2001), such as ellipses, harmonic means and kernel contours (Davidson-Watts *et al.* 2006). Analysis of utilisation distribution discontinuities showed that up to 20% of fix locations increased the size of home ranges disproportionately (Fig. 4.2). Examination of these fixes revealed that they were primarily recorded as bats commuted from roosts to foraging areas and vice versa. Thus 80% cluster cores were used to assess the habitat in which bats were foraging. To account for tracker error in the field, 50 m buffers – based on previous examination of radiotracking accuracy within study areas prior to tagging of bats – were applied to all location fixes when calculating MCPs and cluster core areas.

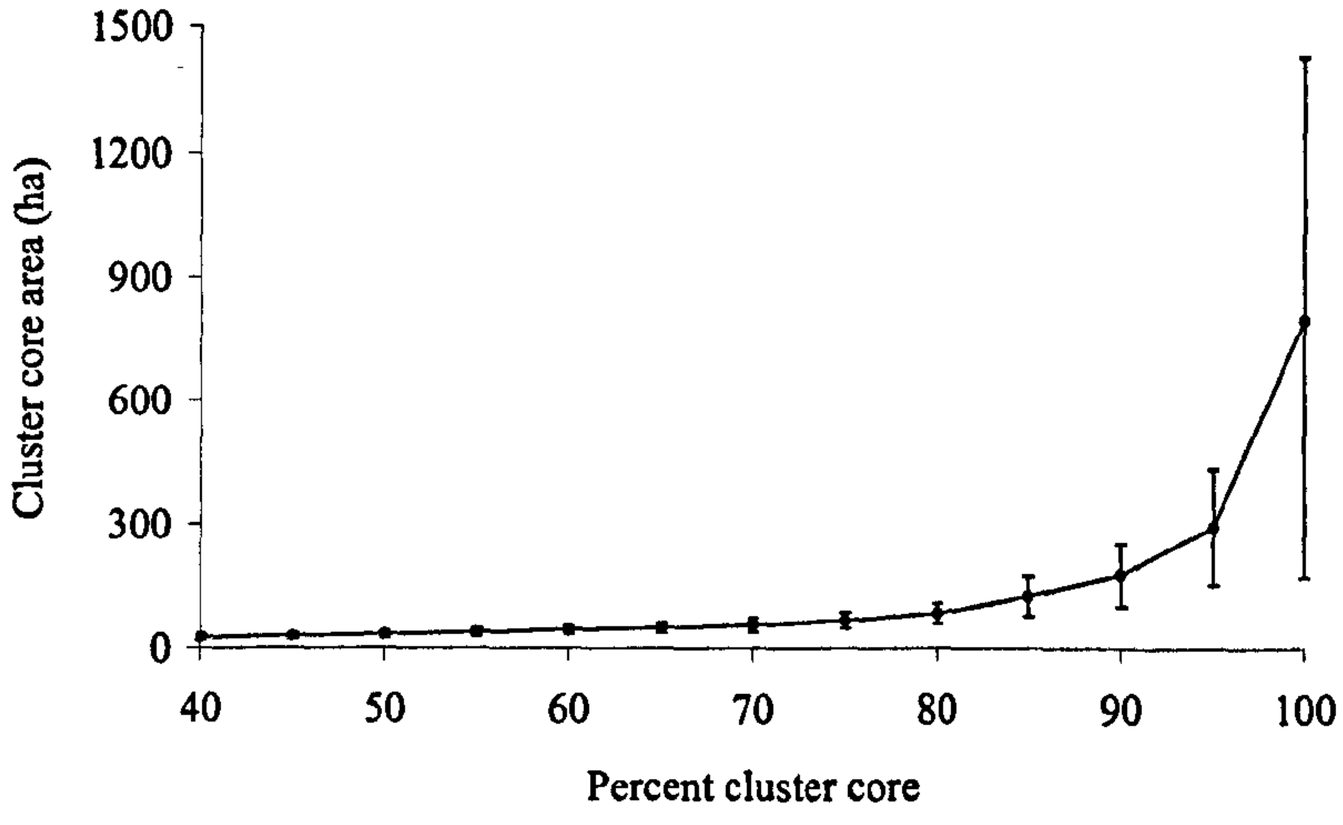

Figure 4.2 – Mean (± SD) utilisation distribution discontinuity for 19 radiotracked *B. barbastellus* (refer to Table 4.2).

Habitat preferences were determined by comparing the habitat composition of areas within which each bat foraged (80% cluster cores) with that available to it (individual MCP home ranges). The used and available habitat compositions were compared using compositional analysis (Compositional Analysis Plus Microsoft Excel tool 6.2, Smith Ecology Ltd, UK) according to the methods outlined by Aebischer *et al.* (1993) to determine initially whether habitats were used in line with availability or if selection was occurring, and secondly to determine the ranking of habitat types. To meet the assumption that n bats > 10 for each study area (Aebischer *et al.* 1993), only data from Houndtor (n = 14 bats) were used in compositional analysis to determine habitat selection.

4.3 Results

Data were obtained from 14 adult female bats at Houndtor (9 post-lactating, 6 non-parous), four at White (2 post-lactating, 2 non-parous), and one at Dendles (non-parous) during May to September, 2007 to 2008. From each bat an average of 185.5 fix locations ± 42.8 *SD* were recorded over a period of 2-4 full nights ($\bar{X}$ = 3.1 nights). Home ranges, foraging areas, and habitat preferences were calculated using data from all radiotracked bats at each site.

Table 4.2 – 100% MCP and 80% cluster core home range areas, and range spans (mean maximum nightly distance from roost to furthest edge of cluster core foraging area) for 19 adult non-breeding (post-lactating or non-parous) female *B. barbastellus* radiotracked in and around south-east Dartmoor National Park, Devon, England between May and September 2007 to 2008.

Bat ID	Site	Home range area (ha)		Range span (km)	Tracking period
		100% MCP	80% core		
190	Houndtor	2895.7	122.8	17.0	Aug-Sep 2007
198		2596.4	107.9	8.7	Jul-Aug 2008
210*		1481.8	77.0	7.9	Aug-Sep 2007-08
240		550.4	65.9	4.7	Aug-Sep 2007
260		4533.0	79.7	20.4	Aug 2008
287		977.5	98.2	5.6	Aug-Sep 2007
310		460.7	56.7	5.4	Aug-Sep 2007
722		1354.6	89.4	8.2	Aug-Sep 2007
739		680.0	57.2	7.2	Aug-Sep 2007
754		2635.3	69.9	11.8	Aug 2008
778		1601.3	99.5	5.0	Sep 2007
860		527.5	65.2	5.1	Sep 2007
882		198.6	57.0	3.2	Jul-Aug 2008
942		1737.5	108.5	8.1	Aug 2008
272	White	491.4	105.9	3.2	Jun 2007
333		2763.2	133.4	12.0	Jul-Aug 2007
808		1829.7	92.1	12.5	Jul-Aug 2008
836		1465.5	85.9	11.9	Aug 2008
672	Dendles	124.3	41.9	1.1	May-Jun 2007
Mean (*SD*)		1521.3 (1152.3)	85.0 (24.8)	8.4 (4.9)	

* Radiotracked in consecutive years of study.

4.3.1 Ranging behaviour

Among all radiotracked bats, maximum range spans (distance from roost to furthest edge of cluster core foraging area) and individual MCP home ranges varied considerably (Table 4.2). On average, bats travelled 8.4 km ± 4.9 *SD* (range 1.1–20.4 km; $n = 19$ bats) from roosts to foraging areas. 80% cluster core foraging areas were relatively constant in size among bats and were dramatically smaller than the individual home ranges through which bats travelled (Table 4.2); on average, foraging areas amounted to only 10.1 % ± 11.7 *SD* of individual home ranges. The pattern of spatial organisation of home ranges and foraging sites among colony members at Houndtor is shown in Fig. 4.3. All bats expressed high site fidelity to foraging areas (Fig. 4.4) with little overlap of foraging areas among colony members (Fig. 4.3b). The only significant overlap of foraging areas among bats was observed when data from consecutive years of tracking were combined (Fig. 4.5).

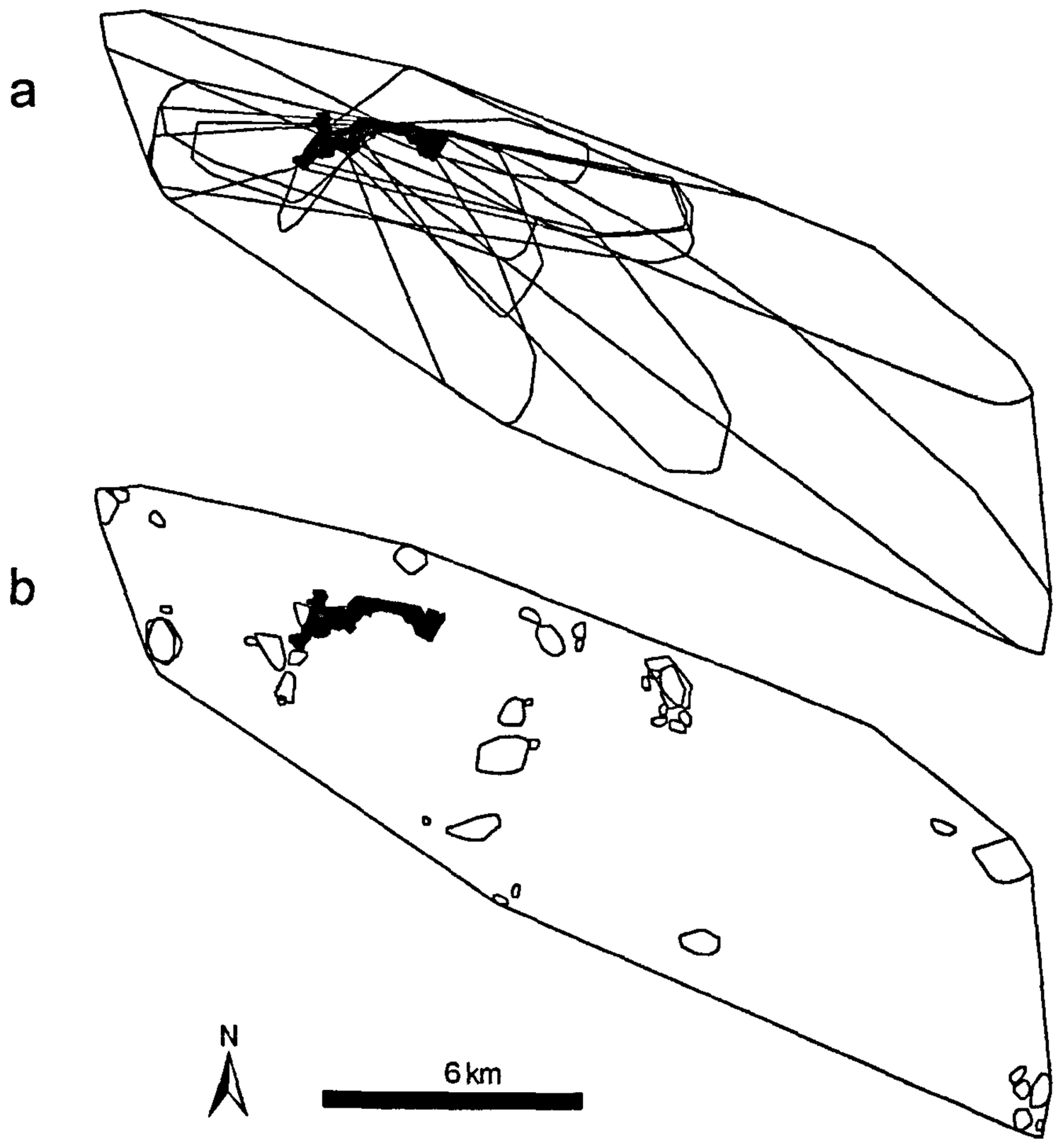

Figure 4.3 – Individual MCP home range areas (a) and 80% cluster core foraging areas (b) encompassed by the combined home range area for 14 adult female *B. barbastellus* radiotracked at Houndtor, Devon, England between May and September 2007 to 2008. Solid grey polygons represent woodland roosting areas.

Figure 4.4 – Examples of foraging site fidelity in four individual *B. barbastellus* (a) to (d). For each of the figures (a) to (c), the foraging areas (80% cluster cores) used by a single bat (ID 287, 882 and 260 respectively, refer to Table 4.2) on repeated nights of radiotracking are displayed as different line shadings. In (d) the foraging areas used by a single bat (ID 210) radiotracked in consecutive years (2007 and 2008, refer to Table 4.2) are displayed.

4.3.2 Habitat availability

The mean composition of available habitats (individual MCP home ranges) at Houndtor over 10307 ha was 39.7% improved grassland, 19.8% broadleaved woodland, 12.8% upland moor, 7% urban, 5% arable, 4.8% riparian, 3.3% unimproved grassland, 3.3% coniferous woodland, 2.6% scrub, 1.3% mixed woodland, 0.4% open water.

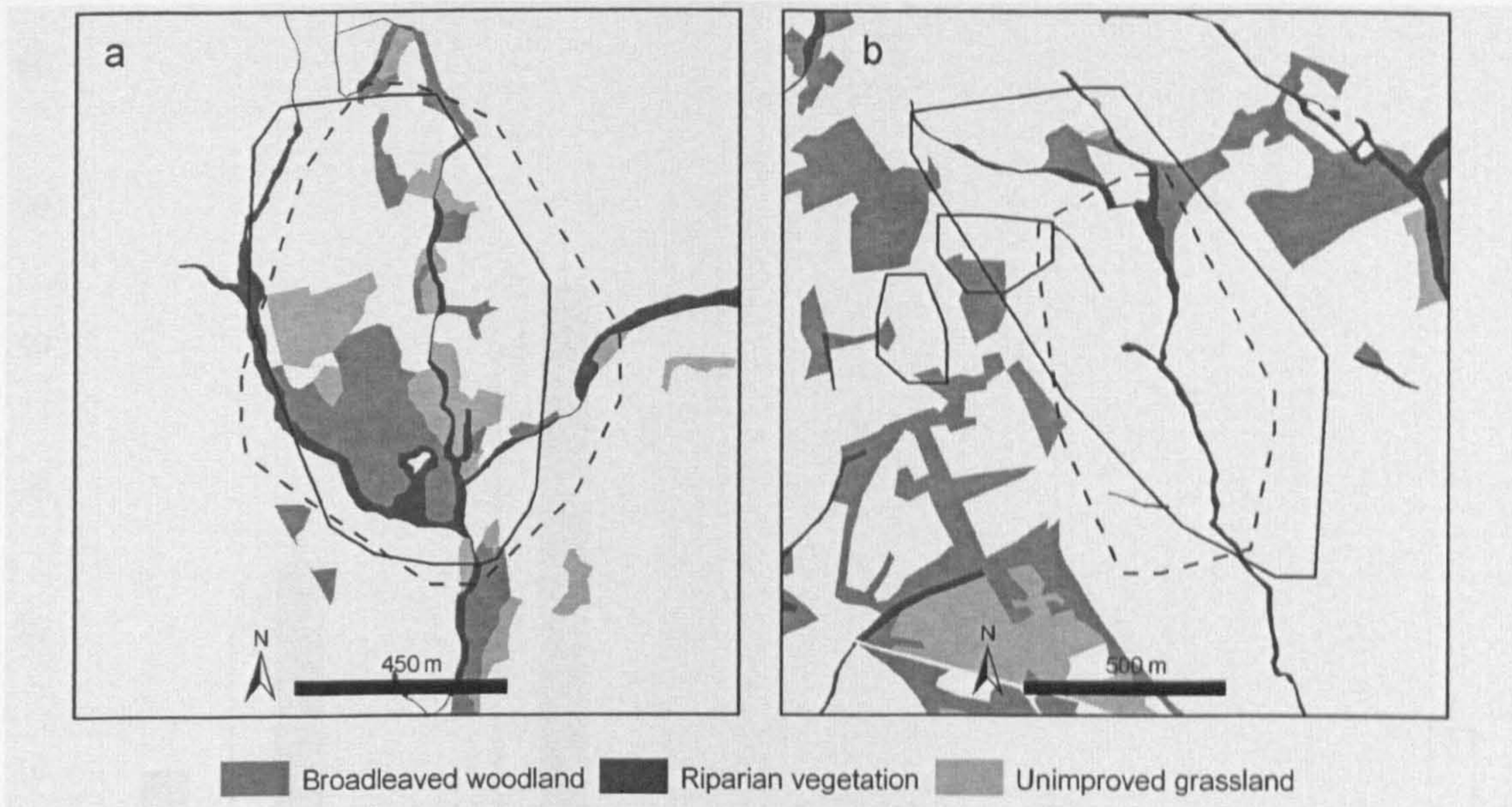

Figure 4.5 – Two examples, (a) and (b), of highly similar 80% cluster core foraging areas among pairs of *B. barbastellus*. In each example, different line shadings are used to show the foraging areas of different bats that were radiotracked in different years.

4.3.3 Habitat selection

The mean percentages of available and used habitats for bats at Houndtor are shown in Fig. 4.6. The composition of habitats used by foraging bats was significantly different from that available (Compositional analysis: weighted mean Wilk's $\Lambda = 0.0167$, $\chi^2 = 57.3221$, d.f. = 10, $P < 0.001$, randomisation $p = 0.005$). A ranking matrix (Table 4.3) ordered habitat types in sequence from most to least selected as follows: riparian>>> broadleaved woodland> unimproved grassland> improved grassland> mixed woodland> coniferous woodland> scrub> urban> open water> arable> upland moor (where a habitat preceding a '>' symbol was preferred to that immediately following the symbol and where a '>>>' symbol shows a significant selection between adjacent ranked habitat categories). Comparisons among all habitat types (Table 4.3) showed that riparian vegetation was selected significantly above all other habitat types except unimproved grassland (where the difference was not significant statistically). Broadleaved woodland was selected significantly over scrub, urban, open water, arable and upland moor. Both grassland categories were selected significantly over urban, open water, arable and upland moor. Open water, arable and upland moor were not significantly selected above any other habitat type and therefore were the habitats least selected by bats.

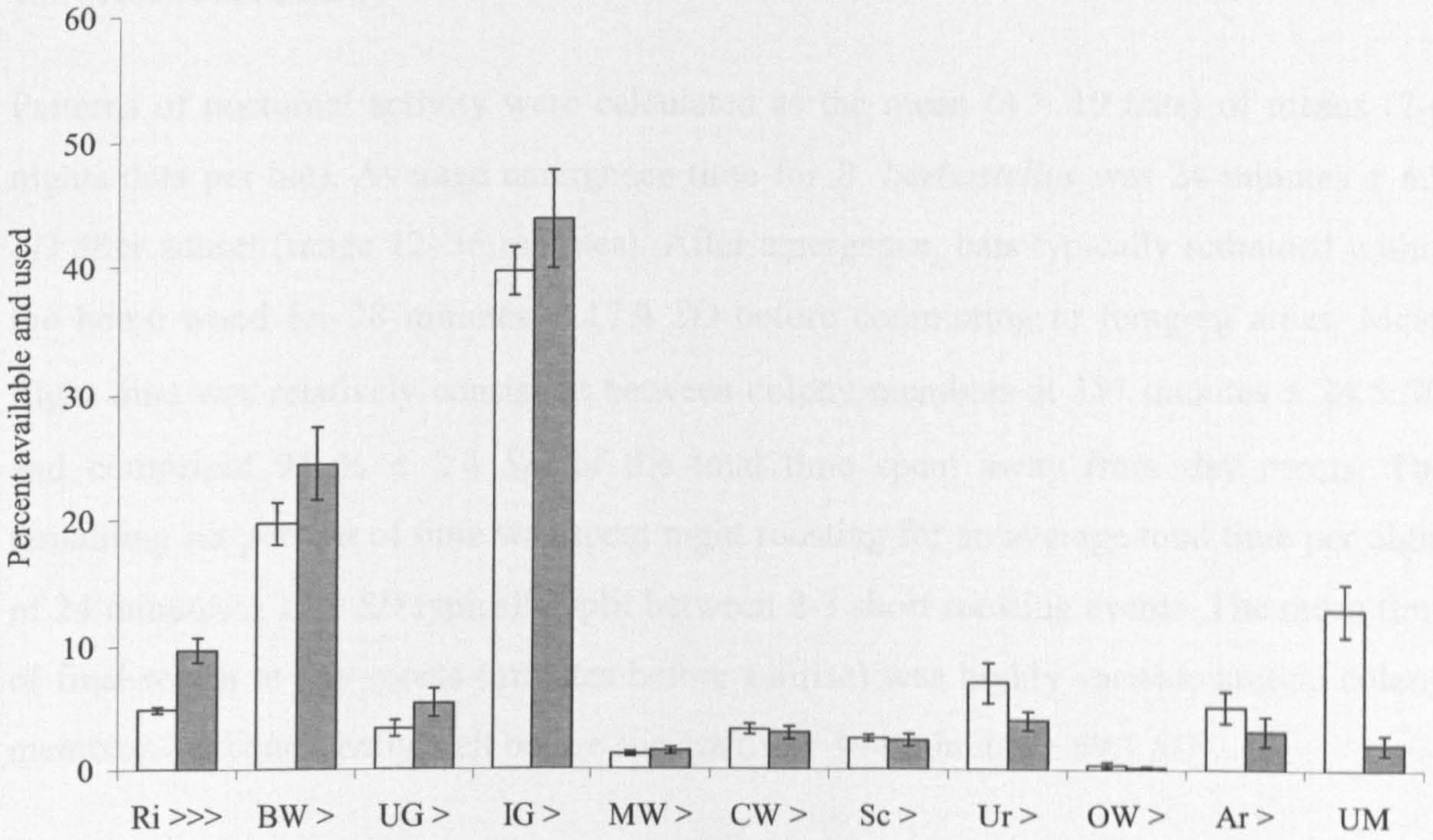

Figure 4.6 – Comparisons of habitat available (100% MCPs; white bars) vs. habitat used (80% cluster; grey bars) (mean percentage area ± SE) for *B. barbastellus* at Houndtor (n = 14 bats). Habitat categories to the left of > are selected over those to the right with >>> showing a significant difference between adjacent habitat types. Habitat abbreviations: Ri Riparian; BW Broadleaved woodland; UG Unimproved grassland; IG Improved grassland; MW Mixed woodland; CW Conifer woodland; Sc Scrub; Ur Urban; OW Open water; Ar Arable; UM Upland Moor.

Table 4.3 – Simplified ranking matrix for *B. barbastellus* at Houndtor (n = 14 bats) based on comparing proportions of habitat within 80% cluster cores (used habitat) and 100% minimum convex polygons (available habitat).

	BW	MW	CW	Sc	UG	IG	Ar	Ri	OW	Ur	UM	Rank
BW		+	+	+++	+	+	+++	---	+++	+++	+++	9
MW	-		+	+	-	-	+++	---	+	+	+++	6
CW	-	-		+	-	-	+++	---	+	+	+++	5
Sc	---	-	-		-	-	+	---	+	+	+++	4
UG	-	+	+	+		+	+++	-	+++	+++	+++	8
IG	-	+	+	+	-		+++	---	+++	+++	+++	7
Ar	---	---	---	-	---	---		---	-	-	+	1
Ri	+++	+++	+++	+++	+	+++	+++		+++	+++	+++	10
OW	---	-	-	-	---	---	+	---		-	+	2
Ur	---	-	-	-	---	---	+	---	+		+++	3
UM	---	---	---	---	---	---	-	---	-	---		0

Table shows habitat preference for each category on every row compared to the corresponding habitat in each column. A significant difference between habitat types is shown by +++ (positively selected) or --- (negatively selected) with + or – showing a non-significant trend. Habitat ranks were calculated by adding the number of + and +++ scores. A rank of 0 signifies the least selected habitat and 10 the most strongly selected habitat. Refer to Fig. 4.6 for abbreviations of habitat categories.

4.3.4 Nocturnal activity

Patterns of nocturnal activity were calculated as the mean (n = 19 bats) of means (2-4 nights data per bat). Average emergence time for *B. barbastellus* was 24 minutes ± 6.9 *SD* after sunset (range 12–36 minutes). After emergence, bats typically remained within the home wood for 28 minutes ± 17.4 *SD* before commuting to foraging areas. Mean flight time was relatively consistent between colony members at 357 minutes ± 24.5 *SD* and comprised 94 % ± 2.4 *SD* of the total time spent away from day roosts. The remaining six percent of time was spent night roosting for an average total time per night of 24 minutes ± 10.9 *SD* typically split between 2-3 short roosting events. The mean time of final return to day roosts (minutes before sunrise) was highly variable among colony members but consistently well before sunrise ($\bar{X}$ = 194 minutes ± 59.1 *SD*).

4.4 Discussion

4.4.1 Home range use and habitat selection

The bats in the present study showed a great deal of individual variation in ranging behaviour, with home range areas varying more than 30-fold. A few bats travelled very large distances to foraging sites (maximum 20.4 km). The reason for why these individuals committed to such long forays is unclear but the behaviour may be indicative of social endeavours beyond the colony, including visits to other maternity colonies to reinforce social bonds among closely related populations, or to male roosts or swarming sites for the purpose of locating mates. Despite their large variation in ranging behaviour, all bats in this study utilised similar sized areas for foraging. In most cases foraging areas formed less than ten percent of individual home ranges, indicating that *B. barbastellus* selects specific sites within home ranges for hunting.

The strong preference for riparian vegetation and broad-leaved woodland was not unexpected. Riparian habitats are known to support high insect densities and their importance to many bat species is well documented (e.g. Russ & Montgomery 2002; Ober & Hayes 2008; Scott *et al.* 2009). Selection of broad-leaved, especially ancient semi-natural, woodland is also common among insectivorous bats (Walsh & Harris 1996; Russ & Montgomery 2002) and in the UK, oak woodland (*Quercus* spp.) in particular has been

found to contain some of the host trees most attractive to moths (Kennedy & Southwood 1984), which dominate the diet of *B. barbastellus* (Chapter 6).

Apart from riparian 'zones' and broad-leaved woodland, unimproved grassland also appears to be an important component of the foraging environment for *B. barbastellus*. Other woodland types are probably only of limited value. Although bats expressed a small preference for improved grassland – by far the most available habitat, characterised *in situ* as small fields bordered by large unkempt hedgerows – field margins, including hedgerows and woodland edges, have been found to support significantly higher moth densities than the agricultural habitats they encompass (Merckx *et al.* 2009a), and bats were often observed hunting there (Zeale pers. obs.). It is likely, therefore, that bats were capitalising on the abundance of a rich hedgerow habitat within home ranges rather than selecting improved grassland *per se*, which is typically species-poor and of limited importance (Walsh & Harris 1996; Russ & Montgomery 2002).

Although linear landscape elements such as hedgerows and treelines are known to be important commuting habitat for numerous species (Limpens & Kapteyn 1991; Verboom & Huitema 1997), they may not necessarily be vital to *B. barbastellus* for this purpose. Radiotracked bats in the present study were frequently observed moving across open ground between foraging sites, and return commutes to roosting areas were fast and direct. It should be noted, however, that outward commutes were considerably slower and more circuitous, and elsewhere bats have been shown to remain faithful to flight paths close to roosts (Greenaway 2004, 2008; Hillen *et al.* 2010), indicating that linear features may facilitate commuting behaviour proximal to woodland roosting areas and probably also provide foraging opportunities prior to reaching core foraging areas. Their use by bats following emergence from roosts may also serve to reduce the risk of predation from aerial-hawking birds (Jones & Rydell 1994; Russo *et al.* 2007).

Urban, open water, arable, and upland moor habitats had the lowest rankings and were therefore the habitats least selected. Although it has previously been suggested that *B. barbastellus* may sometimes feed on insects that aggregate around streetlamps (Zingg 1994; Rydell *et al.* 1996), the negligible use of lit urban areas in this study suggest that such behaviour, if it does occur, may be site-specific and employed rarely elsewhere. Although open bodies of water were not used directly, their role in supporting surrounding riparian vegetation is significant and should be noted. Arable land is arguably the habitat type of least value to bats (Walsh & Harris 1996; Russ & Montgomery 2002),

almost certainly because insect densities there tend to be low as a result of pesticide use and habitat simplification associated with agricultural intensification (Feber *et al.* 1997; Benton *et al.* 2002; Wickramasinghe *et al.* 2004). The extreme exposure to cold temperatures in upland moor habitat is the likely reason for why this habitat type was least preferred by *B. barbastellus*.

Very little overlap of foraging areas was observed among colony members. Hillen *et al.* (2009) suggested that site fidelity rather than territoriality is more important for describing such patterns of spatial organisation in *B. barbastellus*. Provided that the productivity of foraging patches remain stable over time, site fidelity may offer advantages over random foraging in that tradition will avoid the time and energy costs associated with repeated searching for profitable hunting grounds, and would allow individuals to utilise more or less 'private' foraging areas, therefore avoiding territorial confrontation and its associated risks (Chaverri *et al.* 2007; Hillen *et al.* 2009). As was found in Germany (Hillen *et al.* 2009), individual bats in this study expressed high fidelity to foraging sites, both within and between years of study.

The only significant overlap of foraging areas was observed when data from consecutive years were combined, however due to the temporal separation of data it is impossible to determine whether this represents real-time 'sharing' of foraging resources, or whether an individual that occupied a foraging area in the first year had died or moved on before that area was occupied by a second bat. Currently it is unclear how juveniles of most bat species establish foraging areas. While maternal inheritance may be important in some species (e.g. *Myotis bechsteinii*; Kerth *et al.* 2001b), in others, juveniles have been shown to forage independently from their mothers (e.g. *Rhinolophus ferrumequinum*; Jones *et al.* 1995), although kin may share foraging grounds when older (Rossiter *et al.* 2002). Highly similar foraging patches may suggest a shared knowledge of resources, which may be indicative of inheritance; however, more evidence is required to determine if maternal inheritance is important in establishing foraging areas in *B. barbastellus*.

4.4.2 Implications for conservation

Pesticide use and a general shift towards habitat simplification has led to declines in many insect taxa (Wickramasinghe *et al.* 2004; MacLean 2010), however little is known about the effects these declines have had on bats. The rarity and highly specialised diet of *B.*

barbastellus probably make the species particularly sensitive to environmental change (Racey & Entwistle 2003) and the targeting of moths as agricultural and forestry pests in recent years is likely to have been particularly damaging. The fact that some of the only remaining populations in Switzerland exist in remote mountainous areas largely spared by agricultural intensification supports this theory (Sierro 1999). In Britain, rapid declines in common, widespread moths – where numbers of macro-moths caught in light-traps have declined by up to 44% (Conrad *et al.* 2006) – emphasises the scale of the problem. The present study has highlighted the importance of riparian zones and broad-leaved woodland to foraging bats. Unimproved grassland is also likely to be important. These findings are consistent with the hypothesis that *B. barbastellus* select habitats associated with high moth density. Although improved grassland and arable habitats appear to be of little value to the species, hedgerows, tree lines and woodland edges, which are widespread elements of most agricultural landscapes in Europe, may be particularly important where primary habitats are scarce. Their value in facilitating commuting and foraging close to roosting areas should also be considered. Further work is needed to establish the relative value of different field margin biomes to foraging *B. barbastellus*; however participation within agri-environment schemes (AES) and retention of tree cover along hedgerows (which is not currently offered reward under AES) are likely to be beneficial through enhancing moth abundance and diversity and providing greater protection from predators (Russo *et al.* 2007; Merckx *et al.* 2009b). Conversion to organic farming may also increase the abundance of key insect prey and hence improve the use of agricultural areas by *B. barbastellus* (Wickramasinghe *et al.* 2003, 2004).

Significantly, the habitats most important to *B. barbastellus* are some of the least available within agricultural landscapes. Consequently, the main constraint for this species appears to be the relatively uncommon combination of habitats it requires. The data on ranging behaviour suggest management should focus on the protection and enhancement of foraging habitats and linear landscape elements primarily within 8 km of maternity roosts. Given that bats are highly faithful to foraging sites and that site fidelity appears to be an important factor in shaping the spatial organisation of home ranges, foraging sites outside of this range, when identified through radiotelemetry or other means, should also be offered similar protection.

CHAPTER 5

Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces

Material from this chapter has been published as: Zeale MRK, Butlin RK, Barker GLA, Lees D, Jones G (2010) Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, doi: 10.1111/j.1755-0998.2010.02920.x

Abstract

The application of DNA barcoding – taxonomic identification using a short standardized DNA region – to dietary studies allows prey taxa to be identified in the absence of morphological evidence and permits a greater resolution of prey identity than is possible through direct examination of faecal material. For insectivorous bats, which typically eat a great diversity of prey and which chew and digest their prey thoroughly, DNA-based approaches to diet analysis may provide the only means of assessing the range and diversity of prey within faeces. Here, I investigate the effectiveness of DNA barcoding in determining the diets of bat species that specialize in eating different taxa of arthropod prey. For this purpose, a novel taxon-specific primer set was designed and tested, and the performance of short barcode sequences in resolving prey species was examined. Prey DNA was recovered from all faecal samples and subsequent cloning and sequencing of PCR products, followed by a comparison of sequences to a reference database, provided species-level identifications for 149/207 (72%) clones. A phylogenetically broad range of prey was identified in faeces while the detection of non-target groups was completely avoided. In total, 37 unique prey taxa were identified from 15 faecal samples. A comparison of DNA data with parallel morphological analyses revealed a close correlation between the two methods, however, the sensitivity and taxonomic resolution of the DNA method was far superior. The methodology developed here provides new opportunities for the study of bat diets and will be of great benefit to the conservation of these ecologically important predators.

5.1 Introduction

Determining the dietary habits of bats is central to understanding their trophic relationships within ecosystems and is a key part of their conservation management. Because direct observations of feeding events are often impossible, diets of insectivorous bats are studied conventionally by morphological identification of microscopic prey remains, primarily fragments of arthropod cuticle, that remain in faeces (e.g. Beck 1995; Rydell *et al.* 1996; Sierro & Arlettaz 1997). However, the thorough mastication and digestion of prey by bats, coupled with low morphological disparity among related arthropods restricts most practicable taxonomic identifications to order and hence offers only a limited perspective on diet.

Molecular techniques provide alternative approaches to the study of animal diets. Of those described, DNA-based approaches are perhaps the most suitable for examining the range and diversity of prey taken by generalist predators (Symondson 2002). Through polymerase chain reaction (PCR) amplification of DNA sequences unique to prey species, identifications can be achieved even within highly degraded samples such as those found in faeces, gut contents, or regurgitates (King *et al.* 2008). This approach has recently been applied to a range of predator groups, including: marine vertebrates (Jarman *et al.* 2002; Jarman & Wilson 2004; Deagle *et al.* 2005a, b; Parsons *et al.* 2005); seabirds (Deagle *et al.* 2007); marine invertebrates (Blankenship & Yayanos 2005; Braley *et al.* 2009); insectivores (Clare *et al.* 2009); terrestrial invertebrates (Hoogendoorn & Heimpel 2001; Pons 2006; Garros *et al.* 2008); herbivores (Pegard *et al.* 2009; Soininen *et al.* 2009); and to broader studies of trophic ecology (e.g. Carreon-Martinez & Heath 2010, Corse *et al.* 2010).

The successful identification of anonymous DNA sequences to species relies on two important conditions being met. First, sequence divergence at genetic markers must be sufficient to deliver species resolution and second, reference sequences of the same species are required to ensure that an accurate sequence diagnosis can be made. Recent DNA-based diet studies have therefore targeted DNA barcoding regions to achieve high taxonomic resolution (Hebert *et al.* 2003a, b) and to make use of rapidly developing 'barcode' libraries (Barcode of Life Database (BOLD), Ratnasingham & Hebert 2007). In degraded faecal samples however, the propensity for DNA sequences >300 base pairs (bp) to survive digestion can be very low, inhibiting the recovery of full COI barcodes (a

658 bp region of the mitochondrial cytochrome *c* oxidase I (COI) gene) (Deagle *et al.* 2006). Recent examination of 'mini-barcodes' suggests that even very short fragments (100-250 bp) of the complete COI barcode region can deliver 90-95% species-level resolution and are easily recovered from degraded samples (Hajibabaei *et al.* 2006; Meusnier *et al.* 2008). Hence, DNA barcoding using mini-barcode markers can provide an effective solution to obtaining prey species identifications from animal faeces where morphological assessment of prey hard-parts is problematic.

The diversity of prey available to aerial insectivores is considerable. Even bats with 'specialized' diets may consume hundreds of different species. Despite previous publication of numerous arthropod primer sets, to the best of our knowledge none developed thus far can perform as taxon-specific primers for COI barcoding arthropod prey in the faeces of vertebrate predators. In the only DNA-based study of bat diets to date, Clare *et al.* (2009) relied on existing COI barcode primers (LepF1/LepR1, Hebert *et al.* (2004)) to recover DNA sequences from prey fragments individually isolated from faeces. While successful in detecting a large variety of prey species, non-target templates were also amplified, including bacterial, fungal and bat DNA. Although only 3% of sequences were derived from bats the authors noted that other bat species with stronger sequence similarity to primers may cause significant interference, necessitating the development of new primer sets.

The main aim of this study was to develop and validate a universal PCR-based methodology for the study of insectivorous bat diets. For this purpose, very short mtDNA barcode fragments that are expected to remain in degraded faecal samples yet still possess sufficient sequence information to provide species resolution of prey items were targeted. More specifically, the objectives were: (1) to design a novel taxon-specific primer set for the universal amplification of arthropod COI mini-barcodes; (2) to examine nucleotide-sequence divergence at the corresponding mini-barcode marker to test performance in providing species-level diagnoses; (3) to demonstrate a complete working methodology via empirical tests using faecal samples obtained from three diet-differentiated bat species; and (4) to examine the potential for making DNA-based assessments of diet composition by comparing information obtained through conventional and DNA-based diet analyses.

5.2 Materials and methods

5.2.1 Primer design

Primers were designed using COI barcode sequences obtained from GenBank for 11 arthropod orders (10 from Insecta; one from Arachnida) found in the diets of insectivorous bats (Vaughan 1997) and a range of non-target taxa, including bat, bacteria and fungi, that may also be represented within faecal samples (Clare *et al.* 2009). Sequences were aligned in BioEdit (Hall 1999) using ClustalW (Thompson *et al.* 1994) and, where available, arthropod orders were represented by >2 families. Regions of DNA that were conserved among arthropods and had low similarity in non-target taxa were identified as sites for potential primer synthesis. Primers were designed for a number of appropriate binding sites within the full 658 bp barcode region and primer combinations expected to amplify 100-300 bp fragments were tested empirically for specificity using DNA templates purified from a range of target and non-target specimens. After initial screening of unsuccessful primer combinations (i.e. those that amplified from non-target taxa or did not universally amplify arthropod taxa), a single primer set exhibiting the required 'taxon-specific' qualities (Table 5.1) was selected for PCR amplification of arthropod mini-barcodes (157 bp) from faecal samples.

5.2.2 Analysis of marker performance

COI barcodes for all available species in the class Insecta were downloaded from BOLD and trimmed to match the 157 bp marker region to provide a dataset from which examinations of nucleotide-sequence divergence could be made. After removal of sequences that were incomplete or lacked species labels, a final dataset of 38,603 sequences, representing 6867 species, 2669 genera, 260 families, and 23 orders was used to calculate divergence values at each corresponding taxonomic level. For analysis at order level, two orders were selected randomly from the dataset and for each of these an example species was chosen at random. The sequences from these two species were compared and their sequence divergence score calculated as the number of differing bases divided by the aligned sequence length (*p*-distance, Hebert *et al.* 2003a). This sampling was repeated 1000 times in total, choosing two orders at random in each case. The mean, standard deviation and 95% confidence intervals were calculated from the resulting

sample of 1000 divergence scores. This entire sampling process was then repeated at the family, genus, and species levels. For within-species analysis, the same process was applied to comparisons of conspecific sequence pairs. All divergence calculations were performed using a PERL script (PERL 5.8.8 www.perl.org) (constructed by Gary Barker, University of Bristol). To measure the overall resolution of mini-barcodes, sequences from all species present in the dataset were compared and the proportion of non-overlapping barcodes (i.e. barcodes that uniquely identified species) was determined.

Table 5.1 – Taxon-specific PCR primer set developed for this study. Orders successfully amplified by the primer set (Primer specificity: ✓) are shown alongside the relative frequencies with which they occur in chiropteran diets (✓✓✓ frequent, ✓ occasional or rare; Vaughan 1997). The length of the amplified fragment is 157 bp.

Name	Sequences 5' to 3'	
ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	
ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC	
Class: Order	Dietary composition	Primer specificity
Insecta		
Ephemeroptera	✓	✓
Odonata	✓	✓
Plecoptera	✓	✓
Orthoptera	✓	✓
Dermaptera	✓	✓
Hemiptera	✓✓✓	✓
Neuroptera	✓✓✓	✓
Lepidoptera	✓✓✓	✓
Trichoptera	✓✓✓	✓
Diptera	✓✓✓	✓
Hymenoptera	✓✓✓	✓
Coleoptera	✓✓✓	✓
Arachnida		✓
Araneae	✓	✓
Mammalia		
Chiroptera	×	×

5.2.3 Collection of dietary samples

Faeces were collected from 15 bats (11 *Barbastella barbastellus*; 2 *Pipistrellus pipistrellus*; 2 *Myotis nattereri*) caught under license using mist nets and harp traps within woodlands in southern England. These bat species exploit different dietary niches and collectively prey upon a broad range of arthropod taxa (Vaughan 1997). Sampling from

these species therefore provided an opportunity to test primer performance in detecting a phylogenetically diverse group of prey from a variety of DNA matrices. Individual bats were held in sterilised holding bags for a maximum of 30 minutes or until they defecated, after which time they were released. Any resulting faeces were immediately stored in 100% ethanol to preserve DNA samples prior to analysis.

5.2.4 DNA extraction and PCR amplification

For each bat, DNA was extracted from a single faecal pellet weighing 10 to 50 mg ($\bar{X}$ = 27 mg) with the DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions with modifications (Appendix 1). Negative control extractions were performed alongside each batch of extractions from faecal samples to monitor for contamination. All PCRs were carried out in 10 µL volume reactions using the BIOTAQ PCR kit (Bioline). Each PCR contained 1 µL 10x NH4 buffer, 4.55 µL deionized water, 0.4 µL 50 mM $MgCl_2$ solution, 1 µL 2 mM dNTPs, 1 µL forward primer, 1 µL reverse primer, 0.05 µL BIOTAQ DNA polymerase, and 1 µL of DNA template from the final extraction elutions. A touch-down PCR protocol was used to incorporate annealing temperatures of forward (ZBJ-ArtF1c: 56.4 °C) and reverse (ZBJ-ArtR2c: 57.7 °C) primers. The PCR thermal cycling conditions were as follows: 3 min at 94 °C followed by 16 cycles of 30 s at 94 °C, 30 s at 61 °C (decreased by 0.5 °C per cycle) and 30 s at 72 °C followed in turn by 24 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C followed by a final incubation of 10 min at 72 °C. PCR products were visualized on a 1.5% agarose gel and remaining PCR volumes were purified using the QIAquick PCR Purification Kit (Qiagen). Final elution volumes were adjusted for each sample to optimize DNA concentrations for cloning.

5.2.5 Clone library construction and sequencing

PCR products were cloned using the pGEM-T Easy Vector System and high-efficiency competent cells (≥1x10^8 cfu/µg DNA) (Promega). Optimal recombinations were achieved using a 1:1 insert to vector molar ratio and when ligation reactions were incubated overnight at 4 °C. Competent cells were transformed by applying 1 µL of ligation product to 50 µL cells and heat-shocked as follows: 30 min on ice, 25 s at 42 °C (water bath), and 2 min on ice. Colonies containing recombinant clones were selected by X-gal mediated blue/white selection and cultured in 50 µL LB broth at 37 °C for 16-18 h. Cloned inserts

were liberated from cells and amplified via PCR using M13 primers. The resulting amplicons were sequenced bi-directionally using BigDye (version 3.1) chemistry and an ABI3730xl automated sequencer (Applied Biosystems Incorporated).

5.2.6 Identification of DNA sequences

Sequence data were compiled in BioEdit and aligned using ClustalW. Vector and primer sequences were removed and forward and reverse sequences from each clone were compared to identify sequencing error. Sequences were examined further for unexpected insertions/deletions, frameshift mutations, and in-frame stop codons to screen for nuclear mitochondrial pseudogenes (numts), which can be co-amplified with mitochondrial DNA (mtDNA) paralogues and lead to false interpretations of diet composition (Dunshea *et al.* 2008; Song *et al.* 2008; Moulton *et al.* 2009). Other potentially spurious sequences were identified by aligning sequences to authentic mtDNA obtained from BOLD and examining where substitutions occurred in 'suspect' sequences in relation to phylogenetically conserved positions (Dunshea *et al.* 2008). After screening away suspect sequences, all remaining sequences were compared to reference sequences in BOLD to obtain 'closest match' identifications. Identifications to order, family, genus or species were made according to percent similarity of sequence matches. The lower 95% CI for marker divergence at each level of taxonomic affinity were chosen as conservative 'cut-offs' to control for false positive identifications (type I error) (Table 5.2). Accordingly, identifications to order, family, genus or species were made when sequence similarities exceeded 85.9%, 91.0%, 94.9% and 99.3% respectively. Sequences with equal similarity to two or more taxa were identified to the higher taxonomic level that included both taxa.

5.2.7 Morphological vs. DNA-based analysis

DNA extractions from faeces were designed such that all solid faecal material could be retained for additional morphological analysis of hard-parts. Initial examination showed faecal material was not noticeably degraded by extraction processes and an analysis of prey hard-parts could be performed following conventional methods (Whitaker *et al.* 2009). Hard-part fragments were examined under a binocular microscope and compared to reference fragments from vouchered arthropod specimens to allow identification of prey to order. For *B. barbastellus* (n = 11), percent frequency of occurrence (%FO,

proportion of samples containing a given prey taxon) and percent volume (%V, volume of a given prey taxon expressed as a proportion of the total diet) were used to quantify morphological data (Whitaker *et al.* 2009), and %FO and percent clones (proportion of sampled clones containing DNA from a given prey taxon) – which has previously been shown to correspond roughly with known dietary proportions (Deagle *et al.* 2005b) – were used to quantify DNA data. For *Myotis nattereri* and *Pipistrellus pipistrellus* (both *n* = 2) the comparison of morphological and DNA-based diet data was based on presence/absence of prey taxa only. To investigate whether Lepidoptera, Coleoptera and Diptera (the major food of insectivorous bats) could be detected equally within faeces, we fed a single captive brown long-eared bat (*Plecotus auritus*) a series of five treatment meals (comprising waxworms *Galleria mellonella* (Lepidoptera larvae), mealworms *Tenebrio molitor* (Coleoptera larvae) and casters *Calliphora vomitoria* (Diptera pupae)) over a period of 15 days and analysed subsequent faecal samples using both DNA and morphological methods.

5.3 Results

5.3.1 Marker performance

Mean divergences at order, family, genus and species level were 22.5%, 20.0%, 13.6%, and 7.0%, respectively (Table 5.2). At order and family level, all 1000 species pairs could be resolved. At genus level, one species pair (0.1%) could not be resolved and at species level, 22 pairs (2.2%) were unresolved. When all 6867 species in the dataset were compared, 6617 possessed unique mini-barcodes, providing an overall species resolution of 96.4%. Within species, 25.4% of conspecific pairs showed sequence divergence and mean divergence for all 1000 pairs was 0.5%.

5.3.2 DNA-based identification of prey

All faecal samples yielded amplifiable DNA and PCRs produced successful amplifications on all attempts. All PCR amplifications produced single bands and cloning of PCR products typically yielded more than 50 recombinant clones per library. DNA sequencing of 240 clones (16 per faecal sample) produced 215 readable sequences and

Table 5.2 – Divergence values calculated for each taxonomic level within the class Insecta. *n* is the number of different taxa at each level of taxonomic affinity from which example species (or individuals for within-species) were randomly selected, and *n reps* is the number of randomly sampled species (or individuals) pairs that were compared to calculate divergence values. Unresolved branches are the number of sequence pairs with non-unique mini-barcodes (zero divergence).

Taxonomic level	*n*	Mean	SD	Upper 95% CI	Lower 95% CI	*n* reps	Unresolved branches
Order	23	22.5	5.2	32.5	14.1	1000	
Family	260	20.0	6.8	37.0	9.0	1000	
Genus	2669	13.6	5.0	24.9	5.1	1000	1
Species	6867	7.0	4.4	17.2	0.7	1000	22
Within-species	38603	0.5	1.5	3.2	0.0	1000	746

examination of these sequences revealed no indels, frameshift mutations, or in-frame stop codons. Eight sequences showed single base substitutions in phylogenetically conserved positions and were ear-marked as suspected spurious sequences for removal from the dataset. Further examination revealed seven of these 'spurious' sequences matched to prey species that had already been identified within faecal samples. Furthermore, each 'spurious' sequence was only detected once within the complete dataset. Consequently, their removal from the dataset had little effect on the overall assessment of diet. Of the remaining 207 sequences 149 (72%) showed >99.3% similarity to reference sequences on BOLD and were identified to species (or to genus in cases of equal similarity to >1 reference sequence). All other sequences except one ($n = 57$) were at least 95% similar to reference sequences and were therefore identified to genus. The final sequence (94.9% similarity) was identified to family. Overall, 37 different prey taxa were identified from the 15 faecal samples analysed (Table 5.3). Thirty-six of these, represented by 206 sequences (99.5%), belonged to the class Insecta. The final sequence was derived from a spider (Arachnida: Araneae). Crucially, no non-target taxa were detected.

5.3.3 Morphological vs. DNA-based analysis

Of the 15 faecal samples examined, morphological analysis identified prey remains from three distinct insect orders; Lepidoptera, Diptera and Neuroptera. In contrast, seven orders were identified by DNA-based methods (Table 5.4). For all three bat species, prey orders were identified in DNA analyses that were otherwise missed in morphological analyses. For *B. barbastellus*, Lepidoptera was the most dominant prey group. This was evident

Table 5.3 – List of prey identified in the faeces of 11 *Barbastella barbastellus*, 2 *Myotis nattereri* and 2 *Pipistrellus pipistrellus* by DNA analysis; showing percent similarity of closest matches to reference sequences on BOLD. 'Unknown' identifications are provided where similarity scores were not sufficient to permit identification or where reference sequences lacked taxon labels.

Order	Family	Genus	Species ID	% similarity
B. barbastellus				
Araneae	Tetragnathidae	*Metellina*	*Metellina segmentata*	100.0
Diptera	Drosophilidae	*Drosophila*	*Drosophila* sp.	98.7
	Scathophagidae	*Scathophaga*	*Scathophaga stercoraria*	99.4
	Tipulidae	Unknown	Unknown sp.	97.4
Lepidoptera	Arctiidae	*Spilosoma*	*Spilosoma lubricipeda*	100.0
			Spilosoma luteum	100.0
	Geometridae	*Cyclophora*	*Cyclophora punctaria*	100.0
		Ennomos	*Ennomos quercinaria*	100.0
		Odontopera	*Odontopera bidentata*	100.0
		Petrophora	*Petrophora chlorosata*	100.0
	Incurvariidae	*Nematopogon*	*Nematopogon swammerdamella*	99.4
			Nematopogon sp.	98.7
	Lymantriidae	*Calliteara*	*Calliteara pudibunda*	100.0
	Noctuidae	*Agrotis*	*Agrotis exclamationis*	100.0
		Apamea	*Apamea monoglypha*	100.0
		Conistra	*Conistra* sp.	100.0
		Diarsia	*Diarsia* sp.	100.0
		Hoplodrina	*Hoplodrina ambigua*	100.0
		Noctua	*Noctua pronuba*	100.0
			Noctua sp.	100.0
		Ochropleura	*Ochropleura plecta*	100.0
		Orthosia	*Orthosia cerasi*	100.0
		Phlogophora	*Phlogophora meticulosa*	100.0
	Pyralidae	*Plodia*	*Plodia interpunctella*	100.0
Neuroptera	Chrysopidae	Unknown	Unknown sp.	95.7
M. nattereri				
Coleoptera	Carabidae	Unknown	Unknown sp.	96.4
Diptera	Anthomyiidae	*Delia*	*Delia* sp.	99.4
	Chloropidae	Unknown	Unknown sp.	97.9
	Empididae	Unknown	Unknown sp.	96.8
	Syrphidae	*Melanostoma*	*Melanostoma scalare*	100.0
Plecoptera	Perlodidae	Unknown	Unknown sp.	96.3
P. pipistrellus				
Diptera	Tachinidae	Unknown	Unknown sp.	96.4
	Tipulidae	*Limonia*	*Limonia* sp.	96.2
Ephemeroptera	Heptageniidae	*Epeorus*	*Epeorus* sp.	97.4
Lepidoptera	Tortricidae	Unknown	Unknown sp.	94.9
	Gracillariidae	*Cameraria*	*Cameraria ohridella*	100.0
	Incurvariidae	*Incurvaria*	*Incurvaria masculella*	100.0

from both morphological (mean %FO: 100; mean %V: 96) and DNA-based analyses (mean %FO: 100; percent clones (n = 159): 86). Overall, the two diet analysis methods showed a high level of congruency for estimates of diet composition (Table 5.4). Results from the feeding trial confirmed that when faeces contain the remains of lepidopteran, coleopteran and dipteran prey, the primer set is capable of coamplifying DNA from all prey types (Table 5.5).

Table 5.4 – Diet composition of three bat species (*B. barbastellus*, n = 11; *Myotis nattereri*, n = 2; *Pipistrellus pipistrellus*, n = 2) using calculations of percent frequency of occurrence (%FO), percent volume (%V), percent clones, and presence (Y) data for morphological (Morph) and DNA-based assessments of arthropod prey in faeces.

	B. barbastellus				*M. nattereri*		*P. pipistrellus*	
	%FO		# (%) clones	%V				
Prey order	DNA	Morph	DNA	Morph	DNA	Morph	DNA	Morph
Lepidoptera	100	100	137 (86)	96			Y	Y
Diptera	27	27	16 (10)	3	Y	Y	Y	Y
Neuroptera	9	9	5 (3)	1				
Araneae	9		1 (1)					
Coleoptera					Y			
Plecoptera					Y			
Ephemeroptera							Y	

Table 5.5 – DNA and morphological detection of prey in faecal samples collected from a single brown long eared bat (*Plecotus auritus*) during a controlled feeding trial. Starvation periods ensured complete consumption of meals when offered. Meals included: 'mealworm' (larvae of the Mealworm beetle, *Tenebrio molitor*); 'caster' (pupae of the Blue bottle fly, *Calliphora vomitoria*); 'waxworm' (larvae of the Wax moth, *Galleria mellonella*); and 'mixed' (equal parts by volume of each food type).

Day	Treatment	Meal	DNA (number (%) of clones)			Morphological (percent volume)		
			mealworm	caster	waxworm	mealworm	caster	waxworm
1	1	mealworm	3 (100)			100		
2		mealworm	2 (100)			100		
3	Starve							
4	2	mixed	9 (64)		5 (36)	50	30	20
5		mixed	8 (50)	3 (19)	5 (31)	40	30	30
6	Starve							
7	3	mixed	9 (64)	3 (21)	2 (14)	50	40	10
8		mixed	9 (64)	5 (36)		40	40	20
9	Starve							
10								
11	4	caster		3 (100)			100	
12		caster		2 (100)			100	
13	Starve							
14	5	waxworm		3 (100)			60	40
15		waxworm			3 (100)			100

5.4 Discussion

The successful amplification of DNA from the faeces of three insectivorous bat species here supports previous evidence that DNA derived from arthropod prey regularly survives digestion and can be readily detected via PCR (Clare *et al.* 2009). Moreover, through the targeting of a short but informative COI barcode marker a taxonomically broad range of prey was detected and species-level identifications were achieved within all faecal samples. The diagnoses of prey species are likely to be highly robust as, in contrast to other diet studies that have used arbitrary percent similarity criteria to accept species-level identifications, the parameters used for taxonomic assignment in this study were derived directly from calculations of marker divergence among prey taxa. Given that DNA derived from prey taxa may represent only a small fraction of the total DNA in predator faeces (Deagle *et al.* 2006; Vestheim & Jarman 2008), and that bats are readily identified from their own faeces via PCR (Vege & McCracken 2001; Puechmaille *et al.* 2007), the present success in completely avoiding detection of non-target taxa is significant. Moreover, the successful amplification of DNA from spiders, a phylogenetic outlier among insectivorous bat prey, suggests that this primer set is capable of detecting the complete range of arthropod prey in diets.

Despite being one quarter the size, the mini-barcode marker targeted by this primer set showed similar performance in resolving prey taxa to that expected of full-length barcodes. On only three occasions were prey items not resolved at the species level despite having a >99.3% similarity score. In each case the prey sequence matched with equal similarity to two congeneric lepidopteran species, so identifications to genus were made instead. Among all the insects, the lepidopterans pose a particularly challenging case for species diagnosis - they are one of the most taxonomically diverse orders and show lower than average divergence among congeneric species (Hebert *et al* 2003 a, b). Given that the large majority of lepidopteran prey were identified to species, it is expected that other prey groups should be equally, if not more highly, resolved.

Although no clear evidence of mitochondrial pseudogenes was found among prey sequences, numts may be common among insect taxa (Moulton *et al.* 2009) and some numts lack characteristic mutations (including indels, frame-shift mutations and in-frame stop codons) and can be difficult to differentiate from their mitochondrial paralogues (Song *et al.* 2008). Therefore the possibility of numt co-amplification in this study cannot

be completely ruled out. The eight sequences identified as 'spurious' were unlikely numt candidates as in each case they differed by only one base from putative mitochondrial orthologues recovered from the same faeces (numts typically show greater divergence from mitochondrial paralogues (Bensasson *et al.* 2001)). Instead, it is most likely that these anomalous sequences are products of PCR error. Although such errors are largely unavoidable, within any one 157 bp amplicon the probability of incurring a sequence error exceeding one base (0.6%) is low. Consequently, the majority of sequences will still be identified correctly, as single base divergences are a closer reflection of that found among conspecifics (mean: 0.5%) than among congenerics (mean: 7.0%). Indeed, this appears to be true for seven of the eight 'spurious' sequences identified in this study.

Genetic material within faeces is invariably heavily degraded and the recovery of sequences larger than 300 bp may be extremely difficult, if not impossible (Deagle *et al.* 2006). This is likely to be true of insectivorous bats, which chew and digest their prey thoroughly. By targeting a short (157 bp) multi-copy mtDNA marker, success was had in amplifying prey templates from all faeces that we tested, irrespective of sample quality. Previous studies have noted prey-specific biases in DNA survival during digestion (e.g. Deagle & Tollit 2007). If this is true for bats, it would be reasonable to predict that larger, hard-bodied prey that are disproportionately well represented in solid faecal material, would be preferentially detected. In this study, the detection of small, soft-bodied micro-moths among larger, more heavily sclerotised prey suggests that if a bias in DNA survival does occur, this method still provides adequate sensitivity to detect those prey items most vulnerable to digestion.

The three bat species sampled in this study occupy distinct dietary niches. *B. barbastellus* specialises in eating lepidopteran moths taken by aerial hawking; *P. pipistrellus* takes mainly aquatic Diptera, also by hawking; and *M. nattereri* takes almost entirely diurnal Diptera gleaned from their nightly resting places. Typically however, the diets of all these species contain a broad range of prey orders (reviewed in Vaughan 1997). The clone libraries generated for each of these species closely reflect previous descriptions of diet. All of the prey items we identified are typical of what might be encountered by these bat species, suggesting that our analysis appears not to have been confounded by interference from secondary predation (Sheppard *et al.* 2005).

A comparison of DNA data with those obtained via morphological analysis shows that a greater diversity of prey was detected via DNA-based analyses. Currently, DNA-

based approaches to diet analysis provide only limited scope for making quantitative interpretations of diet composition (King *et al.* 2008). The use of clone libraries to estimate prey proportions represents one possible interpretation that has previously been shown to correspond roughly with known dietary proportions (Deagle *et al.* 2005b). The assessment of clone libraries for *B. barbastellus* in this study provided an estimate of diet composition that closely resembled that calculated from morphological data, both of which corresponded well with previous diet studies for this species (Rydell *et al.* 1996; Sierro & Arlettaz 1997). Whether or not the same level of congruence can be achieved for large sample sizes or for different bat species with more complicated diets remains to be seen. Meanwhile, other techniques such as quantitative PCR and high-throughput pyro-sequencing are also proving well suited to dietary analysis and may provide more useful interpretations of diet composition (Valentini *et al.* 2009; Deagle *et al.* 2009; Soininen *et al.* 2009). However, in the absence of a robust method for quantifying faecal DNA, a combination of both DNA-based and conventional techniques is likely to prove most beneficial (Casper *et al.* 2007; Braley *et al.* 2009).

The present success in resolving nearly all prey items to the genus or species level is testament to the role DNA barcoding can play in significantly improving our understanding of animal diets. Barcode libraries are of course an integral part of the equation, and current campaigns to further develop these resources will greatly benefit future diet studies. In summary, this PCR-based method provides an efficient non-invasive tool for providing robust prey species identifications in the diets of insectivorous bats. Moreover, this method may be universally applicable across a broad spectrum of vertebrate insectivores. For insectivorous bats, DNA barcoding of faeces currently provides the only realistic means of determining the range and diversity of prey within diets. As such, this method offers new perspectives and opportunities in the study of bat dietary ecology and predator-prey interactions, and may contribute significantly to the conservation of these ecologically important predators.

CHAPTER 6

Prey selection and diet composition

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Abstract

The barbastelle, *Barbastella barbastellus*, is considered to be a predator that specializes in eating moths; however, diet studies using conventional approaches to faecal analysis (i.e. morphological classification of prey remains) have been unable to determine which moth taxa are taken. Consequently, important questions remain unanswered regarding the species' dietary requirements and trophic ecology, including whether eared prey are eaten. Molecular approaches to diet analysis offer new perspectives on species ecology by greatly improving the resolution to which prey items can be identified. In this study, polymerase chain reaction (PCR) and DNA barcoding – species identification using a short standardized DNA region – are used alongside conventional morphological techniques to obtain unprecedented information on prey selection by, and the trophic relationships of, *B. barbastellus*. In total, 877 prey mini-barcode sequences were recovered from the faeces of 51 bats. By comparison with reference arthropod barcode sequences, 815 (93.0 %) prey sequences were identified to species. The remainder were identified either to genus or to family. Although a broad range of prey was identified, of a total 89 unique prey taxa recorded within faeces 75 (84%) belonged to the order Lepidoptera. Species from the families Noctuidae and Geometridae were the prey types eaten most frequently by bats. Seventy-three (82%) prey species were from families known to employ some form of hearing-based defense against echolocating bats. In light of this new evidence, hypotheses of prey selection and predator-prey interactions for *B. barbastellus* are addressed, and implications for conservation are discussed.

6.1 Introduction

Information on predator-prey interactions is essential for understanding a wide variety of biological questions ranging from animal behaviour and population dynamics to the direct and collateral impacts that humans have on ecosystems. However, for insectivorous bats, which take small prey and whose foraging behaviour is difficult to witness directly, observing these interactions is especially difficult. Analysis of faecal contents allows indirect measures of prey selection to be made and historically has contributed a great deal to understanding the evolutionary and ecological processes that shape bat-prey relationships. Despite this, conventional approaches to diet analysis – i.e. morphological identification of prey hard parts – limit understanding of prey selection to what is visibly discernable within faecal samples, thus most prey items can only be identified to the level of order (Vaughan 1997). Novel molecular approaches to diet analysis provide new perspectives on trophic relationships by offering much improved detection and resolution of prey, and are therefore revolutionizing studies of animal ecology (Symondson 2002; King *et al.* 2008; Chapter 5). For insectivorous bats, predator-prey interactions can now readily be characterised at the species level (Clare *et al.* 2009; Zeale *et al.* 2010).

The relationship between echolocating bats and insects that possess hearing-based defenses is one of the most intensively studied examples of predator-prey adaptation in biology (Fullard 1982; Surlykke 1986; Jones & Waters 2000; Fullard *et al.* 2003a, 2003b; Denzinger *et al.* 2004). However, although ears evolved in many insect taxa together with evasive flight as antipredator adaptations, it is debatable whether bats have coevolved counter-adaptations against eared prey (Waters 2003; Rydell *et al.* 1995; Jones & Rydell 2003). For bats and their insect prey, the allotonic frequency hypothesis proposes that some bat species responded to hearing prey by calling at frequencies outside the range of the prey's greatest auditory sensitivity (Fullard 1998). Despite much support for this hypothesis, other benefits could have initially driven selection for these changes, such as increased detection distance at low frequencies or improved spatial resolution at high frequencies (Rydell *et al.* 1995). The low amplitude calls used by many foliage gleaning bats represents another widely debated example of counteradaptation by bats to eared prey (Faure *et al.* 1990; Faure & Barclay 1992), but again there are alternative benefits associated with this behaviour, such as reduced echo-interference from background clutter (Schnitzler & Kalko 1998; Jones & Rydell 2003).

The barbastelle, *Barbastella barbastellus*, has one of the narrowest diets of all Palaearctic bat species, taking almost exclusively moths by aerial-hawking (Beck 1995; Rydell et al. 1996; Sierro and Arlettaz 1997). Tympanal organs (ears) are present in many lepidopteran families, however *B. barbastellus* echolocates at frequencies (33 kHz peak frequency) well within the best hearing range of most moth species (typically 20-50 kHz) and is not known to glean prey from surfaces (Sierro & Arlettaz 1997; Denzinger *et al.* 2001), suggesting that an alternative adaptive strategy to those already hypothesised may be used by this species to counter moth hearing. Crucially though, not all moths have ears, and because soft-bodied (heavily digested) moth prey are not readily identifiable below order level using conventional morphological approaches to faecal analysis, it remains unclear whether eared moths are taken by *B. barbastellus* in any great number.

In this study, I use the polymerase chain reaction (PCR) and DNA barcoding – species identification using a short standardized DNA region – to make species diagnoses of prey in the faeces of *B. barbastellus* (Zeale *et al.* 2010; Chapter 5) and to determine the extent to which eared prey feature in the diet. In light of this new information, prey selection and diet composition are discussed in the context of echolocation and prey defenses to examine whether counteradaptations may have evolved in *B. barbastellus* that bypass the defensive adaptations in moths (i.e. whether coevolution in its strictest sense (Janzen 1980) has occurred). Given the extent of its dietary specialisation, *B. barbastellus* may be particularly vulnerable to negative changes in the availability of its prey. Therefore, in addition, I discuss the conservation implications of the species' foraging tactics amidst the backdrop of large declines in moth populations recorded in the UK in recent history (Conrad *et al.* 2006; Fox *et al.* 2010).

6.2 Materials and methods

6.2.1 Collection and analysis of dietary samples

Faecal pellets were collected from *B. barbastellus* caught under license throughout southern England and Wales between March and October 2005 to 2009 (Table 6.1). Individual bats were held in sterilised holding bags for a maximum of 30 minutes or until they defecated, after which time they were released. All faeces produced were placed immediately in 100% ethanol to preserve samples prior to analysis. Diet composition was

Table 6.1 – Source of *B. barbastellus* faecal samples used for DNA and morphological assessment of diet.

Location	Date	Year	*n* bats	*n* faecal pellets
Devon	May–September	2007–2008	19	27
Dorset	June	2007	1	4
Hampshire	March–August	2005–2007	7	7
Isle of Wight	April–October	2006–2009	19	22
Pembrokeshire	August	2008	1	1
West Sussex	July–August	2008	4	4

assessed using both DNA barcoding and conventional morphological techniques following the methods detailed in Chapter 5. In summary, prey DNA was extracted from faeces using DNA Stool Kits (Qiagen Ltd., Crawley, West Sussex, UK) and amplified via PCR using the ZBJ-primer set (Table 5.1). PCR products were cloned using the pGEM-T Easy Vector System (Promega Corp., Madison, WI, USA) to isolate individual amplicons (157 base pair fragment of the cytochrome *c* oxidase subunit I (COI) gene) for DNA sequencing. On average 15.5 clones ± 2.2 *SD* from each faecal pellet were sequenced. The complete sequence dataset was screened for the presence of nuclear mitochondrial pseudogenes (numts) and sequence anomalies resulting from PCR and sequencing error which can inflate estimates of dietary diversity (Dunshea *et al.* 2008; Song *et al.* 2008). Suspected 'spurious' sequences were removed and all remaining sequences were compared to reference sequences in the Barcode of Life Database (BOLD, Ratnasingham & Hebert 2007) to obtain 'closest match' identifications. Identifications to order, family, genus or species were made when prey sequences showed greater than 85.9%, 91.0%, 94.9% and 99.3% similarity to reference sequences on BOLD, respectively (conservative percent cut-offs were determined from calculations of marker divergence, see *5.2.2* and *5.3.1*). Lepidopteran prey were classified as eared or non-eared according to previous descriptions of auditory organs among lepidopteran families (Scoble 1992; Fullard 1998, and references therein). Following DNA extractions, all solid faecal material was examined under a binocular microscope (GX XTL–2I, 7–42x magnification; GM Optical, Suffolk, UK) for a parallel morphological assessment of prey remains. All undigested diagnostic prey hard-parts were identified at the family level with the aid of published keys (Shiel *et al.* 1997; Marian James, unpubl.) and by comparing recovered fragments with vouchered arthropod specimens.

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6.2.2 Data analysis

For DNA data, percent frequency of occurrence (%FO; proportion of samples containing a given prey taxon) and percent clones (proportion of sampled clones containing DNA from a given prey taxon) were used to describe diet composition (Deagle *et al.* 2005b Zeale *et al.* 2010; Chapter 5). Frequency histograms of the number of prey operational taxonomic units (OTUs; i.e. haplotype prey sequences) recovered per bat and the recovery of specific OTUs within the cumulative diet (n = 51 bats) were plotted, and a species accumulation curve with 95% confidence intervals (based on 50 random resampling efforts) was calculated using EstimateS (Colwell 2006). For morphological data, %FO and percent volume (%V, volume of a given prey taxon expressed as a proportion of the total diet) were used to describe diet composition (Whitaker *et al.* 2009). Percent volume of each prey type within each faecal pellet was estimated to the nearest 5% (Jones 1990), except where prey items constituted only 1-2 % of any sample, in which case they were recorded as such. The total percent volume for each prey type was calculated as the average value from all samples (n = 51 bats). To determine whether dietary diversity varied seasonally (between March-April (n = 6), May-June (n = 10), July-August (n = 29), and September-October (n = 6)), or between the gender (male, n = 8; female, n = 43) or reproductive state of adult female (breeding (pregnant or lactating), n = 12; non-breeding (post-lactating or non-parous), n = 30) bats, Simpson's index of diversity was calculated for bats in each group and comparisons between groups were made using Kruskal-Wallis and Mann-Whitney tests. Simpson's index is calculated here as 1–D, where $D = \Sigma p_i^2$ and where p_i is the proportion of the ith prey family in the diet. Index values range from 0 (no diversity) to 1 (infinite diversity). In all tests, significance was set at $P < 0.05$.

6.3 Results

6.3.1 Identification of prey

Prey DNA was amplified from all faecal samples and DNA sequencing of 1008 clones produced 910 readable sequences. Thirty-three of these sequences showed single base substitutions in phylogenetically conserved positions, were earmarked as suspected

spurious sequences resulting from PCR error, and were subsequently removed from the dataset. Each of these sequences matched to prey species that had already been identified within corresponding faecal samples and most were detected only once within the complete dataset; hence their removal had little effect on the overall assessment of diet composition. Screening for indels, frame-shift mutations and in-frame stop codons revealed no evidence of numt coamplification. Of the remaining 877 sequences, 815 (93.0%) showed >99.3% similarity to reference sequences on BOLD and were identified to species (or to genus in cases of equal similarity to >1 reference sequence). Forty-five sequences (5.1%) were identified to genus; 17 sequences (1.9%) were identified to family. In total, 89 different prey OTUs were identified (Table 6.2). Importantly, no non-target taxa (bat, bacteria, fungi) were detected in any of the samples. Most prey OTUs were detected only once among samples (n = 51 bats), however, a few were recovered repeatedly (Fig 6.1a). The noctuid moth species *Cosmia trapezina* (Dun-bar); *Apamea*

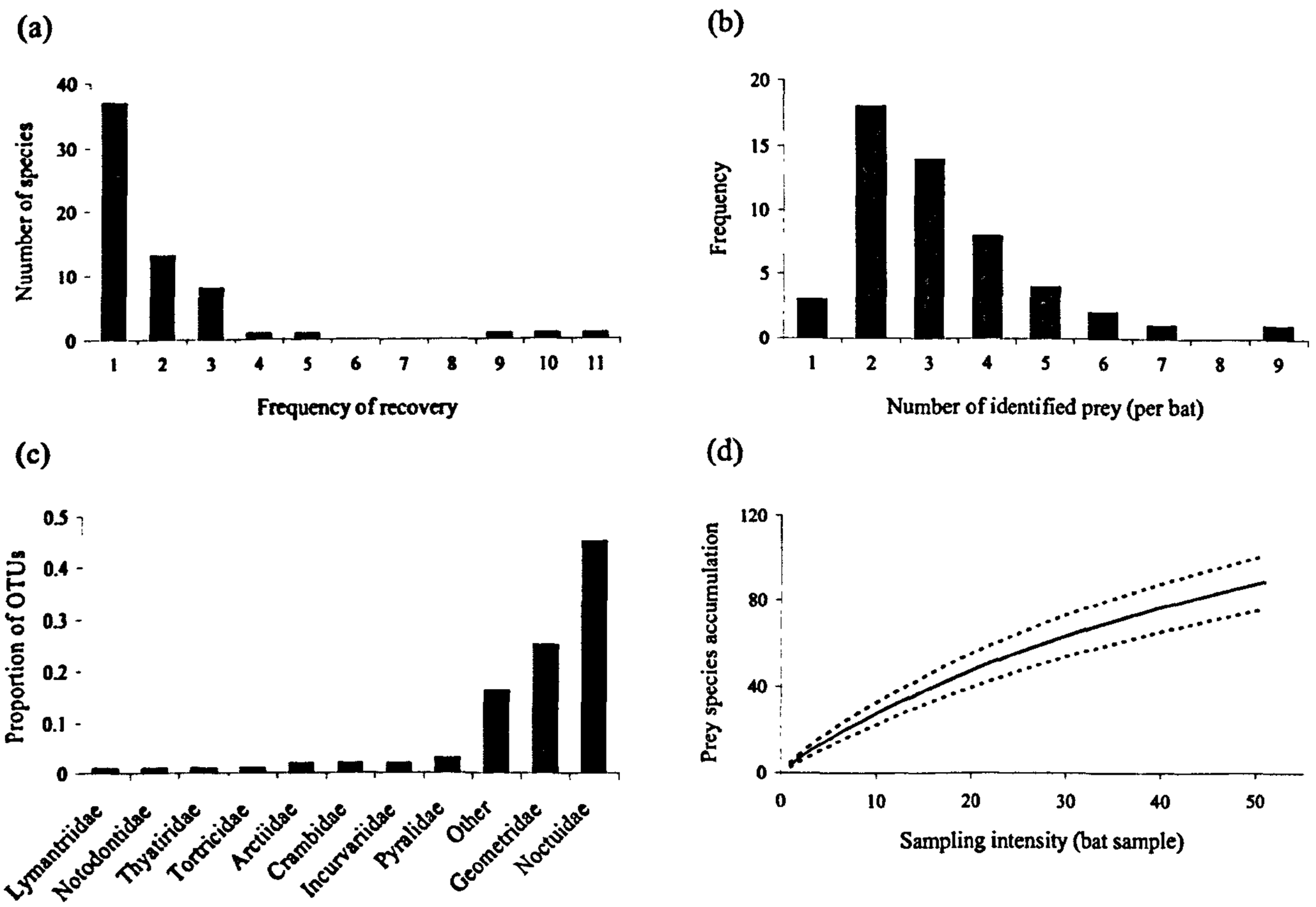

Figure 6.1 – Analysis of DNA data recovered from the faeces of 51 *B. barbastellus* caught between March and October 2005 to 2009 throughout southern England and Wales: (a) frequency with which individual prey species were recovered from faeces; (b) number of prey operational taxonomic units (OTUs) recovered from individual bat faecal samples; (c) proportion of prey OTUs (n = 89) identified within faeces according to lepidopteran family of origin, 'Other' includes all non-lepidopteran prey OTUs (refer to Table 6.2); (d) accumulation curve (with 95% confidence intervals (dashed lines), based on 50 random resampling efforts) of prey OTUs recovered from faeces with increased sampling intensity.

monoglypha (Dark Arches); and *Noctua pronuba* (Large Yellow Under-wing) were identified within the faeces of eleven, ten and nine bats respectively and were the prey species most frequently recovered. The frequency of recovery (number of bats in which the prey OTU was detected) and the frequency of detection (total number of clones identified) of each prey OTU individually are displayed in Table 6.2. On average 3.2 prey OTUs ± 1.6 *SD* were recovered from each faecal pellet (Fig. 6.1b). The species accumulation curve (Fig. 6.1d) indicates that the 89 prey OTUs identified in the cumulative diet represent only part of the complete diversity of prey taken by *B. barbastellus*.

6.3.2 Prey selection and diet composition

Although a taxonomically broad range of prey was detected, moths were by far the most common and abundant prey group (Table 6.3). This was evident from both DNA and morphological results which, overall, showed a high level of congruency (Table 6.3). Cumulatively, bats preyed on moths from 10 different families; however, the large majority of species taken were from the families Noctuidae and Geometridae (Fig. 6.1c). Individually, the faeces of 49/51 bats (96%) contained predominantly moths and is reflected by the low Simpson's index diversity score (0.13) for the cumulative diet (n = 51 bats). Only in two bats was the consumption of moths not apparent. In these samples, tipulid flies were the dominant prey item identified. Thirty-four bats (67%) were considered to have eaten only moths during the period of hunting associated with each Faecal pellet – i.e. no morphological or DNA-based evidence of non-lepidopteran prey was detected. Examination of dietary diversity between seasonal periods, and between male and female bats and breeding and non-breeding females revealed no significant difference for any of the comparison made – moths dominated the diet in all of the groups analysed. Of the 75 identified moth prey taxa taken by bats, 67 (89%) were identified as larger 'macro' moths (see wingspan data in Table 6.2) and 72 (96%) belonged to lepidopteran families known to employ some form of hearing-based defense against bats. Overall, eared prey – including moths and lacewings (Neuroptera: Chrysopidae) – were identified in 96 % of samples and constituted 811/877 (92%) of the clones that were sequenced (Table 6.3). Individual species possessing hearing organs are identified in Table 6.2.

Table 6.2 – List of prey identified in the faeces of 51 *Barbastella barbastellus* by DNA analysis; showing percent similarity of closest matches to reference sequences on BOLD. 'Unknown' identifications are provided where similarity scores were not sufficient to permit identification or where reference sequences lacked taxon labels. Common names are provided for species where available. Wingspans are provided for Lepidoptera as an indicator of body size (Waring & Townsend 2009). Presence of auditory organs (•) indicates prey items known to employ some form of hearing-based defence against echolocating bats, as previously described for Lepidoptera (Scoble 1992; Fullard 1998, and references therein) and Neuroptera (Miller 1970; Miller & Olesen 1979). Total *n* clones identified = 877.

Order	Family	Species	Common name	Wingspan (mm)	Auditory organs	Recovery (*n* bats)	Frequency (*n* clones)	% similarity
Araneae	Agelenidae	Unknown sp.				1	4	94.2
	Tetragnathidae	*Metellina segmentata*	Lesser Garden Spider			1	1	100.0
Diptera	Drosophilidae	*Drosophila* sp.				3	11	98.7
	Limoniidae	Unknown sp.				1	2	99.4
	Scathophagidae	*Scathophaga stercoraria*	Yellow Dung-fly			2	4	99.4
	Tachinidae	*Tachina* sp.				1	1	97.8
	Tipulidae	*Tipula fulvipennis*				3	15	100.0
		Tipula helvola				1	14	100.0
		Tipula sp.				3	5	99.4
Hemiptera	Gerridae	Unknown sp.				1	2	93.6
Hymenoptera	Ichneumonidae	Unknown sp.				1	1	97.2
Lepidoptera	Arctiidae	*Spilosoma lubricipeda*	White Ermine	34-48	•	2	31	100.0
		Spilosoma luteum	Buff Ermine	28-40	•	3	38	100.0
	Crambidae	*Eudonia mercurella*		16-19	•	1	5	100.0
		Udea prunalis		20-24	•	1	4	100.0
	Geometridae	*Acasis viretata*	Yellow-barred Brindle	25-29	•	1	1	99.4
		Cabera pusaria	Common White Wave	25-28	•	2	5	100.0
		Campaea margaritata	Light Emerald	30-40	•	2	2	100.0
		Chloroclysta truncata	Common Marbled Carpet	24-30	•	1	1	100.0
		Cyclophora linearia	Clay Triple-lines	26-33	•	1	3	100.0
		Cyclophora punctaria	Maiden's Blush	18-25	•	1	2	100.0
		Ennomos fuscantaria	Dusky Thorn	35-40	•	3	8	100.0
		Ennomos quercinaria	August Thorn	42-50	•	1	3	100.0
		Epirrhoe alternata	Common Carpet	20-25	•	1	2	100.0
		Eupithecia assimilata	Currant Pug	17-22	•	1	3	100.0
		Eupithecia exiguata	Mottled Pug	20-22	•	2	4	100.0
		Idaea aversata	Riband Wave	23-30	•	5	36	100.0

Table 6.2 – *Continued*

Order	Family	Species	Common name	Wingspan (mm)	Auditory organs	Recovery (*n* bats)	Frequency (*n* clones)	% similarity
		Lomographa bimaculata	White-pinion Spotted	22-26	•	1	16	100.0
		Odontopera bidentata	Scalloped Hazel	32-40	•	3	17	100.0
		Opisthograptis luteolata	Brimstone Moth	32-37	•	3	8	100.0
		Peribatodes sp.			•	1	1	100.0
		Petrophora chlorosata	Brown Silver-line	31-37	•	1	21	100.0
		Petrophora sp.			•	1	1	98.7
		Selenia dentaria	Early Thorn	28-40	•	1	2	100.0
		Selenia sp.			•	1	1	98.7
		Xanthorhoe designata	Flame Carpet	25-28	•	1	1	100.0
		Xanthorhoe fluctuata	Garden Carpet	18-25	•	2	12	100.0
	Incurvariidae	*Nematopogon swammerdamella*		18-21		1	2	99.4
		Nematopogon sp.				1	2	98.7
	Lymantriidae	*Calliteara pudibunda*	Pale Tussock	40-60	•	1	4	100.0
	Noctuidae	*Acronicta psi*	Grey Dagger	30-40	•	1	5	100.0
		Agrochola circellaris	The Brick	33-38	•	1	1	100.0
		Agrochola lychnidis	Beaded Chestnut	30-35	•	1	1	100.0
		Agrotis exclamationis	Heart and Dart	30-40	•	2	24	100.0
		Agrotis puta	Shuttle-shaped Dart	30-32	•	2	19	100.0
		Agrotis segetum	Turnip Moth	27-40	•	3	10	100.0
		Agrotis sp.			•	1	1	98.7
		Anaplectoides prasina	Green Arches	40-50	•	1	1	100.0
		Apamea monoglypha	Dark Arches	45-55	•	10	91	100.0
		Apamea sp.			•	2	2	99.3
		Autographa gamma	Silver Y	35-40	•	1	4	100.0
		Caradrina morpheus	Mottled Rustic	32-38	•	2	5	100.0
		Caradrina sp.			•	1	1	98.7
		Colocasia coryli	Nut-tree Tussock	27-35	•	1	1	100.0
		Conistra sp.			•	3	15	100.0
		Cosmia trapezina	The Dun-bar	25-33	•	11	92	100.0
		Cosmia sp.			•	1	1	98.5
		Craniophora ligustri	The Coronet	30-35	•	1	4	100.0

Table 6.2 – *Continued*

Order	Family	Species	Common name	Wingspan (mm)	Auditory organs	Recovery (*n* bats)	Frequency (*n* clones)	% similarity
		Craniophora sp.			•	1	2	98.7
		Diarsia sp.			•	2	20	100.0
		Dichonia aprilina	Merveille du jour	35-40	•	1	13	100.0
		Eupsilia sp.			•	1	1	98.9
		Hoplodrina ambigua	Vine's Rustic	32-34	•	4	28	100.0
		Ipimorpha retusa	Double Kidney	26-32	•	1	1	99.4
		Mythimna sp.			•	2	2	98.8
		Noctua comes	Lesser Yellow Underwing	37-45	•	1	2	100.0
		Noctua fimbriata	Broad-bordered Y. U-wing	45-55	•	1	2	100.0
		Noctua pronuba	Large Yellow Underwing	45-55	•	9	55	100.0
		Noctua sp.			•	10	64	100.0
		Ochropleura plecta	Flame Shoulder	25-30	•	1	10	100.0
		Orthosia cerasi	Common Quaker	34-40	•	2	14	100.0
		Orthosia munda	Twin Spotted Quaker	38-44	•	1	5	100.0
		Panolis flammea	Pine Beauty	30-33	•	1	2	100.0
		Phlogophora meticulosa	Angle Shades	45-50	•	3	28	100.0
		Phlogophora sp.			•	1	1	98.7
		Rivula sericealis	Straw Dot	18-22	•	1	1	100.0
		Xestia c-nigrum	Setaceous Hebrew Character	35-42	•	2	7	100.0
		Xestia sexstrigata	Six-striped Rustic	36-38	•	2	2	100.0
		Xestia xanthographa	Square-spot Rustic	32-35	•	1	1	100.0
		Unknown sp.			•	3	11	100.0
	Notodontidae	*Pterostoma palpina*	Pale Prominent	35-55	•	1	2	99.4
	Pyralidae	*Conobathra repandana*		20-25	•	1	1	100.0
		Plodia interpunctella	Indian Meal Moth	14-20	•	2	3	100.0
		Plodia sp.			•	1	2	98.7
	Thyatiridae	*Thyatira batis*	Peach Blossom	32-38	•	1	1	100.0
	Tortricidae	*Choristoneura* sp.				1	1	98.0
Neuroptera	Chrysopidae	*Chrysoperla* sp.			•	2	9	95.7
		Unknown sp.			•	5	11	94.9
Trichoptera	Limnephilidae	*Potamophylax latipennis*				1	1	99.4

Table 6.3 – Diet composition of *B. barbastellus* (n = 51 bats) using calculations of percent frequency of occurrence (%FO), percent clones, and percent volume (%V) for morphological (Morph) and DNA-based assessments of arthropod prey in faeces.

	%FO		# (%) clones	%V
Prey order	DNA	Morph	DNA	Morph
Insecta				
Lepidoptera	96	96	796 (91)	93
Diptera	18	25	52 (6)	4
Neuroptera	12	20	20 (2)	2
Coleoptera	0	4	0 (0)	<1
Trichoptera	2	2	1 (<1)	<1
Hymenoptera	2	2	1 (<1)	<1
Hemiptera	2	2	2 (<1)	<1
Arachnida				
Araneae	4	2	5 (<1)	<1
Eared prey[†]	96		811 (92)	

[†] Prey items known to employ some form of hearing-based defence against echolocating bats, according to previous descriptions of auditory organs among lepidopteran (Scoble 1992; Fullard 1998, and references therein) and neuropteran (Miller 1970; Miller & Olesen 1979) families.

6.4 Discussion

6.4.1 Prey selection and diet composition

The detection of almost exclusively nocturnal flying prey in the diet supports previous suggestions that *B. barbastellus* hunts predominantly by aerial-hawking (Rydell *et al.* 1996; Sierro & Arlettaz 1997). As was also found by Rydell *et al.* (1996), the rare occurrence of spiders and small fragments of plant material in faeces indicates that bats may also occasionally glean prey from vegetation, although this material could also have been ingested in roosts and spiders may also be hawked while suspended from silk threads. In the absence of any robust observations of gleaning behaviour, it currently remains unclear whether *B. barbastellus* employs this strategy to any significant extent.

Moths are clearly a fundamental component of the diet for *B. barbastellus*; however, prior to this study, few data were available on specifically what types of moth are taken (Beck 1995; Rydell *et al.* 1996; Sierro and Arlettaz 1997). Using molecular techniques, I provide the first robust evidence that *B. barbastellus* feeds almost exclusively on eared moths. This prompts the question how these bats are able to bypass the sensory defenses of their prey. New evidence shows that *B. barbastellus* uses a stealth echolocation strategy by emitting low intensity calls (10 to 100 times lower in intensity than those of

other aerial-hawking bats) that exploit the relative difference in hearing thresholds between predator and prey (Goerlitz *et al.* 2010). The calls appear to be sufficiently quiet that *B. barbastellus* remains undetected even when close; hence evasive flight maneuvers are probably not elicited in prey. Goerlitz *et al.* (2010) concluded that the low intensity calls impose the cost of reduced prey detection distance to the bat with no compensating benefit other than making its calls inconspicuous to eared prey; hence, this strategy appears to be the first conclusive example of a coevolved counter-adaptation in bats to the hearing-based defenses of prey. From an ecological perspective, this strategy clearly places *B. barbastellus* at a considerable advantage over, and reduces competition with, other aerial-hawking bat species that emit calls of higher intensity, whose diets typically contain less than 30 percent moths by volume (Vaughan 1997).

The question remains though why *B. barbastellus* does not take non-moth prey more frequently. Evidence from both DNA and morphological analyses shows bats are clearly capable of preying successfully on a broad range of insect taxa other than moths. Indeed, two faecal samples contained no evidence of moth consumption whatsoever. Perhaps non-moth prey are rarely encountered at feeding places, although this seems unlikely because the riparian habitats preferred by foraging *B. barbastellus* (Chapter 4) typically support an abundance of both moth and non-moth prey types (Peterson *et al.* 2004; Naiman *et al.* 2005; Fukui *et al.* 2006; Chinery 2007). As was postulated by Schober & Grimmberger (1987), the relatively narrow mouth and weak teeth of *B. barbastellus* may explain why highly chitinous (hard) insects, such as beetles, are almost never found in the diet. However, many insect taxa other than moths are soft-bodied and hence should presumably be available to bats. Perhaps *B. barbastellus* has become so efficient at catching eared moths, which may be favoured as highly nutritious prey, that other less profitable prey items can largely be neglected.

On finding no evidence of large moths in faeces, Sierro & Arlettaz (1997) inferred that smaller moths (wingspan < 30 mm), which were abundant at feeding sites, probably constitute the majority of moth prey items taken by *B. barbastellus*. Moreover, they suggested that *B. barbastellus* may be somewhat restricted in the size of prey that it can capture by its relatively slender skull morphology (Schober & Grimmberger 1987). In this study, the majority of prey items identified by DNA were large moths from the families Noctuidae and Geometridae, showing conclusively that *B. barbastellus* is not restricted to taking only small prey items. This result was supported by the recovery of numerous

large-moth fragments (legs, eggs, antennae, tarsi) from faeces. Because optimal foraging theory predicts that animals should select prey that maximize the net rate of energy return (Stephens & Krebs 1986), presumably larger moths should be taken preferentially when they are available to bats. Importantly, however, it is not currently clear whether *B. barbastellus* is capable of distinguishing moths by size. The low intensity and short pulse duration of calls, and low-duty-cycle echolocation used by *B. barbastellus* may suggest that bats are not sufficiently informed of prey characteristics to enable selection of larger, more profitable moths accurately (Roverud *et al.* 1991; Jones & Rydell 2003). If true, the relative abundance of large and small moths in the diet should reflect the availability of each prey type at feeding sites. Currently however, datasets comparing diet composition with simultaneous assessments of prey availability at feeding sites are lacking, therefore it remains unclear whether *B. barbastellus* conforms to predictions of optimal foraging models when hunting moths.

6.4.2 Implications for conservation

The high level of dietary specialisation exhibited by *B. barbastellus* probably makes the species particularly vulnerable to negative changes in the availability of its prey (Sierro & Arlettaz 1997; Racey & Entwistle, 2003). In the UK, there is now dramatic evidence of a severe decline in moth numbers (Conrad *et al.* 2006; Fox *et al.* 2010), raising immediate concern over the implications this has for the persistence of *B. barbastellus* populations. Most notably, in southern Britain – where *B. barbastellus* appears to be restricted to (Chapter 2) – numbers of large 'macro' moths caught at light traps have declined by over 40 % and is probably indicative of a much wider biodiversity crisis (Fox *et al.* 2010). Of the moth species taken by bats, *Spilosoma lubricipeda*[1] (white ermine) and *Ennomos fuscantaria* (Dusky Thorn) have shown the greatest declines – 77% and 98% respectively over the period 1968-2002 (Fox *et al.* 2010).

Some of the apparent decline in moths is almost certainly attributable to considerable changes in land use and the enormous increases in pesticide use associated with agricultural intensification since the mid 1900's (Tilman *et al.* 2001; Robinson & Sutherland 2002; Foley *et al.* 2005; MacLean 2010). Clearly there is a very urgent need to

[1] Although a number of arctiid moth species produce ultrasonic clicks to avoid capture by bats (Fullard *et al.* 1979; Bates & Fenton 1990; Miller 1991), *Spilosoma* spp. are not known to exhibit this behaviour, which may explain why they are present in the diet while other arctiid species are not (Table 6.2).

address this alarming trend, not only for the invertebrate species concerned and the higher predators that depend on them but also to ensure that overall ecosystem stability is maintained. Determining the extent of the threat to *B. barbastellus* and how best to manage and mitigate it will require a better understanding of the species' dietary plasticity as well as the effects that climate change is predicted to have on both bat and invertebrate populations (McCarty 2001; McLaughlin *et al.* 2002; Rebelo *et al.* 2010). Although it is encouraging that some of the moth species taken by bats have shown considerable increases in abundance in recent history (e.g. *Hoplodrina ambigua* (Vine's Rustic) – 413 %) and some *Noctua* spp., e.g. *Noctua fimbriata* (Broad-bordered Yellow Underwing – 954 %)), the overall diversity of moths encountered by bats at feeding sites is probably declining (Conrad *et al.* 2006; Fox *et al.* 2010). This is a concern because individually, moth species tend to show acute temporal fluctuations in abundance, thus reductions in moth diversity will almost certainly result in bats having to cope with a less stable, more unpredictable, food resource. Individuals may be forced to search for prey over larger areas, increasing pressure on energy budgets. This may be particularly problematic for *B. barbastellus* which shows high fidelity to foraging sites (Chapter 4).

In areas where *B. barbastellus* is present, habitats should be managed to support both the abundance and diversity of moth populations. Where maternity colonies are identified, the abandonment of insect control measures within associated woodlands is a priority. In bat foraging areas, and more generally within 8 km of roosting areas (Chapter 3), the protection and enhancement of habitats associated with high moth abundance is essential, including the retention of hedgerows, tree lines, and woodland edge habitats, particularly within agricultural landscapes where these features are likely to be a fundamental component of the foraging environment for *B. barbastellus* (Chapter 4; Merkx *et al.* 2009a, 2009b,). Participation in agri-environment schemes (AES), including organic practice, is also likely to be beneficial (Merkx *et al.* 2009b; Wickramasinghe 2004, 2005).

This study provides further evidence of the significant improvements that can be made in understanding bat diets and trophic ecology when molecular techniques are applied to diet analyses. The success that was had in detecting and amplifying prey DNA, and in identifying the majority of prey items to species, confirms this methodology to be an efficient tool for providing robust prey species identifications in the diets of insectivorous bats.

CHAPTER 7

General Discussion

7.1 Summary of findings

In response to human-generated environmental change, many bat species have shown population declines and range contractions (Hutson *et al.* 2001; Racey & Entwistle 2003). It is well established that bats are keystone species within their biological communities, providing essential ecosystem services and playing important roles in maintaining overall ecosystem stability (Nowak 1994; Racey & Entwistle 2003). Thus, current declines in bat populations are likely to be indicative of a wider biodiversity crisis. Indeed, bats have enormous potential as bioindicators, although currently this potential remains largely unexplored (Jones *et al.* 2009). For many threatened species, a current lack of information regarding their distribution and ecological requirements places significant constraints on the level of protection that can be provided through conservation measures. This is particularly true of rare taxa, whose rarity and difficulty of detection make obtaining informative sample sizes a significant challenge. Despite this, advances in several fields, including species distribution modelling and molecular biology, as well as technological developments in survey equipment (e.g. lightweight radio-telemetry tags and acoustic lures) continue to improve the accessibility of rare bats to conservation biologists. By embracing many of these disciplines, and by integrating newly developed techniques with more established ones, this study has made a significant contribution to understanding the behaviour, ecology and conservation requirements of one of Europe's rarest bat species.

The use of predictive modelling is assuming greater relevance in conservation biology as a tool for providing rapid, cost-effective assessments of species' distributions and for identifying areas of high conservation value (Papeş & Gaubert 2007; Rebelo & Jones 2010). In this study, presence-only models proved extremely useful for identifying new *B. barbastellus* maternity colonies and provided further confirmation of the importance of native mature woodland to this species (Greenaway 2001; Russo *et al.* 2004). A key concern raised by model predictions is that perhaps as much as 90-95% of woodland areas in the UK are unsuitable for supporting *B. barbastellus* maternity colonies. In fact, this is probably a conservative estimate because many features of woodlands that influence their suitability could not be modelled. The apparent lack of suitable woodland habitat is a major limiting factor for *B. barbastellus*, not only in the UK but also elsewhere in Europe (Russo *et al.* 2004); thus, improving woodland areas to suit observed habitat preferences should be a major conservation priority for this species.

Although the models were clearly very useful in facilitating the search for maternity colonies, only when model predictions were combined with a concerted field-based effort was it possible to identify the specific roosts used by bats. In particular, the acoustic lure proved invaluable for obtaining bats in the hand that could then subsequently be tagged and radiotracked to locate roosts. Nearly all of the free-flying *B. barbastellus* caught in this study were done so with the aid of the lure. Thus, most of the results presented here are directly attributable to the success of this tool and highlights the importance of using a variety of techniques to maximise data collection from rare species.

B. barbastellus shows roosting preferences that should be regarded as key points in conservation protocols (Russo *et al.* 2004, 2005; Hutson *et al.* 2001). Many of the preferences identified in this study are the same as those identified elsewhere in Europe (Russo *et al.* 2004, 2005) suggesting that the management recommendations made here can be applied with confidence throughout the species' range. Primary among these is the promotion of ancient woodland characteristics, including the preservation of large numbers of dead and decaying trees. In addition to the findings of Russo *et al.* (2004), the results of this study highlight the importance of woodland close to water and suggest that any tree supporting a suitable cavity, independent of tree characteristics, may be used by *B. barbastellus*. Thus, where woodlands are managed to support this species, it is advisable that a conservative approach is taken to diagnosing 'suitable' trees, such that any tree supporting a cavity should be considered of conservation value. Ideally, this will include any sizeable cavity, located anywhere on trees, because in this study there was no apparent selection of cavities according to any of the characteristics measured and currently it remains unclear what constitutes a 'suitable' cavity for *B. barbastellus*. Furthermore, the availability of a variety of cavity features within woodland roosting areas is likely to be important for providing bats with a diversity of different roost microclimates that can be exploited according to ambient conditions. It is recommended that further work is carried out to determine in greater detail the cavity preferences of *B. barbastellus* (e.g. the size, structure and internal microclimate of roost cavities) so that more comprehensive guidelines for woodland management can be provided.

The analysis of foraging habitat requirements in this study is the most detailed of its kind to date for *B. barbastellus* and the first to identify statistically the selection of specific habitat types. It is now clear that riparian zones and broadleaved woodland are the most important habitat types to this species. Given that the diet of *B. barbastellus*

appears to be consistent throughout Europe – i.e. dominated by moths (Beck 1995; Rydell et al. 1996; Sierro and Arlettaz 1997) – the protection and management of these habitat types to enhance moth abundance and diversity will have universal benefits throughout the species' range. Both riparian zones and broadleaved woodland are key foraging habitats for many bat species (Walsh & Harris 1996; Russ & Montgomery 2002); thus, managing landscapes to enhance these habitats for *B. barbastellus* will have wider benefits for bat fauna in general. The high fidelity of bats to foraging areas suggests that individual *B. barbastellus* may be relatively inflexible in where they can forage. Indeed, site fidelity appears to be important in maintaining the spatial organisation of foraging areas among conspecifics (Hillen *et al.* 2009). Consequently, when identified, it is important that foraging areas are protected and managed to enhance the availability of moth prey. In agricultural landscapes, agri-environment schemes may be beneficial for the conservation of *B. barbastellus*. Organic farming and schemes that encourage the management of linear landscape elements to enhance invertebrate populations will most likely provide the greatest benefits. Ensuring high water quality is also essential for maintaining the productivity of riparian habitats for bats. Improving tree cover along flightlines close to woodland roosting areas will also be beneficial (Greenaway 2008).

Traditionally, difficulties associated with identifying arthropod prey to below the level of order have been an important limitation for understanding how best to manage landscapes to suite the dietary requirements of bats. Even on rare occasions when diagnostic prey fragments can be recovered from faeces, identifications below the family level are usually extremely difficult without specialist knowledge of arthropod morphology and taxonomy. This study has shown that molecular approaches to diet analysis offer much improved resolution of prey and can be implemented with relatively little training. The development of a new, highly efficient taxon-specific primer set was a significant achievement, and it is expected that this tool will be used widely in future diet studies of bats and other insectivores. Indeed, it has already been used successfully to study the diets of African free-tailed bats, *Chaerephon pumilus* and *Mops condylurus* (family: Molossidae) (Bohmann *et al.* unpubl.), and to identify the importance of mosquito pest species in the diets of Australian bats (Gonsalves *et al.* unpubl.). It is also currently being used to identify the insect prey of grey and brown long-eared bats, *Plecotus austriacus* and *Plecotus auritus* (Razgour *et al.* unpubl.), and common dormice, *Muscardinus avellanarius* (O'Reilly *et al.* unpubl.). The DNA barcoding approach that

was used in this study was also pivotal to the advances that were made. The extensive databases of arthropod barcode sequences currently available are an invaluable resource for identifying prey items with high taxonomic resolution and current campaigns to expand these databases will continue to increase their value for molecular ecology research. Although the DNA barcoding concept was originally established to aid taxonomists, its value in identifying unknown DNA sequences from environmental samples such as predator faeces is a significant spin-off application and highlights the significant gains that can be had from integrating resources from different disciplines. The use of bacterial cloning and Sanger sequencing in this study proved to be an effective method for making assessments of bat diets; however, due to the continuous and rapid advancement of molecular technologies, this method has already largely become obsolete. For the same cost, newer high-throughput sequencing techniques can provide many orders of magnitude more sequence data and do not require a cloning stage within methods, which is relatively time consuming and expensive. Because of the enormous volumes of sequence data that can be generated, modern sequencing techniques (e.g. 454 next generation sequencing) allow much improved detection of rare prey items and provide more meaningful quantitative assessments of diet composition (Valentini *et al.* 2009; Deagle *et al.* 2009; Soininen *et al.* 2009). It is essential that conservation biologists exploit these new and emerging techniques to ensure that the most effective management strategies can be implemented.

Prior to this study, the diet of *B. barbastellus* was one of the least known among Palaearctic bats. By combining novel molecular methods with conventional approaches to diet analysis, this study has provided the most detailed assessment of the species' diet to date. Indeed, the diet of *B. barbastellus* is now one of the best known among European bats. The results showed that the performance of the molecular technique far exceeded that of the conventional approach and has provided the first species-level identifications of prey for this species. The discovery that *B. barbastellus* feeds almost exclusively on eared moths is significant and has allowed, for the first time, hypotheses of predator-prey coevolution to be tested conclusively (Goerlitz *et al.* 2010). Current declines in UK moth populations are a major concern for the conservation of many bat species, but particularly so for *B. barbastellus*. It is not currently known what degree of flexibility *B. barbastellus* possesses in its prey choice, but determining this will be important for evaluating the threat to the species from declines in its preferred moth prey. In any case, addressing

current trends in UK moth populations is fundamentally important for maintaining the stability of many ecosystems and should be considered a major conservation priority. In areas where *B. barbastellus* is present, both moth abundance and diversity should be encouraged to ensure that a stable and reliable food source is available for the species throughout the year. Using the species identifications of prey made in this study it is now possible to manage habitats for *B. barbastellus* with much greater specificity, such as preserving and promoting the specific food plants of moth species found in the diet.

7.2 Final Remarks

Multidisciplinary approaches are essential in conservation biology to ensure that all aspects of an animal's biology that govern its conservation needs can be studied and understood. By embracing different disciplines and new tools and techniques this study has made a significant contribution to understanding the conservation biology of *B. barbastellus*. One of the major successes was the discovery of six new maternity colonies, increasing the number of known colonies in the UK by 40 %. During the last four years, the total number of colonies identified has more than doubled, primarily due to improvements in survey techniques and a better understanding of this species' ecology. The increasing rate with which *B. barbastellus* is being detected suggests that the species is probably not as rare in the UK as previously considered. However, the species still exhibits natural rarity, sparse distributions, small breeding populations, and very high ecological specialisation, all of which inflate the vulnerability of the species to environmental change. Consequently, *B. barbastellus* must remain a conservation priority species. Lack of suitable woodland and declining prey populations are the major conservation concerns for this species, both of which need to be addressed with some urgency to prevent further declines in numbers and to ensure the persistence of the species in the future. In conclusion, the findings of this study provide the most detailed account of habitat use to date as well as the most extensive analysis of diet. These findings will make significant contributions to the development of more comprehensive management plans for this rare species.

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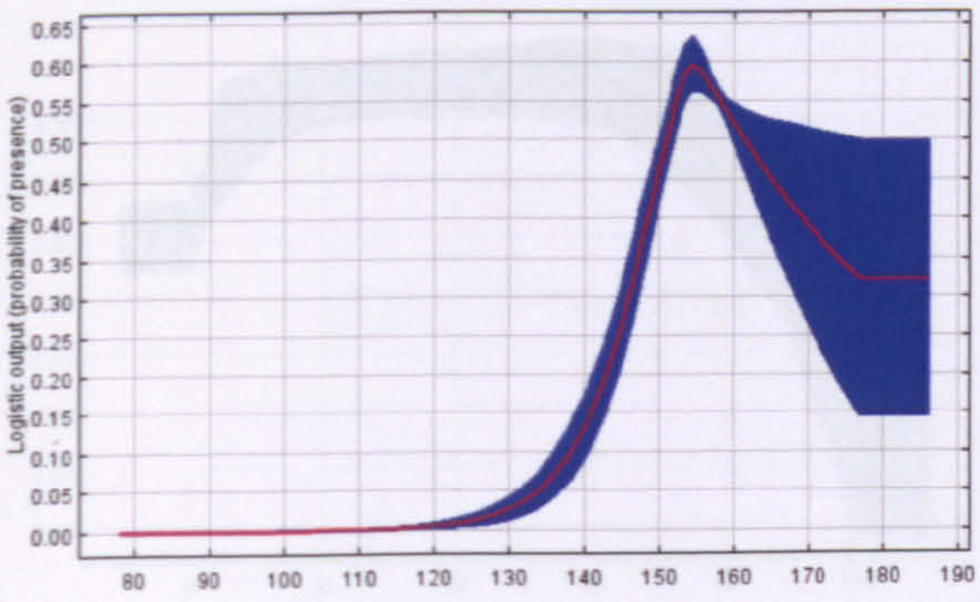
APPENDIX A

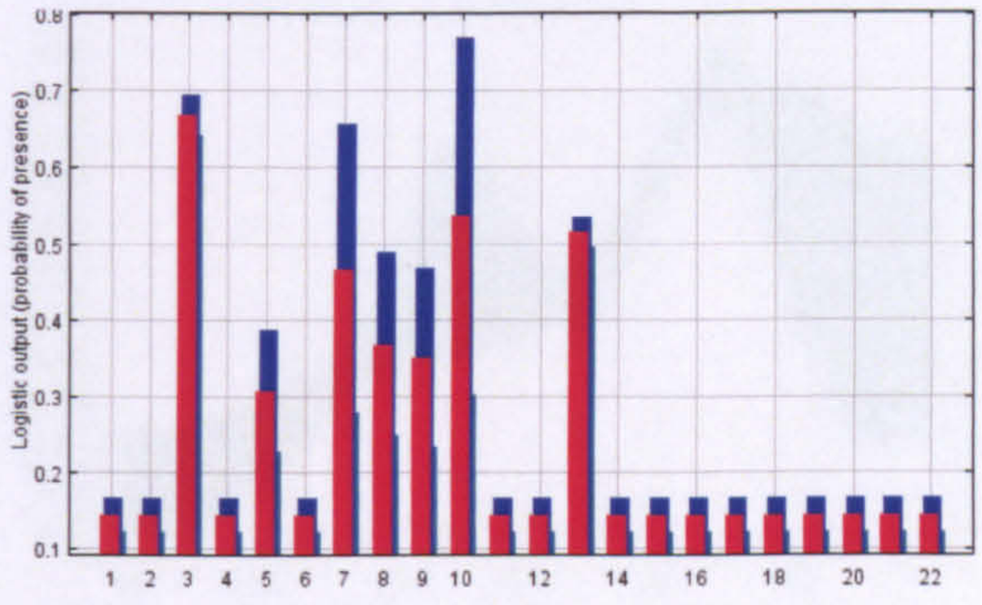
Probability of presence plots from model calculations of *B. barbastellus* distribution in the UK

Mean summer temperature



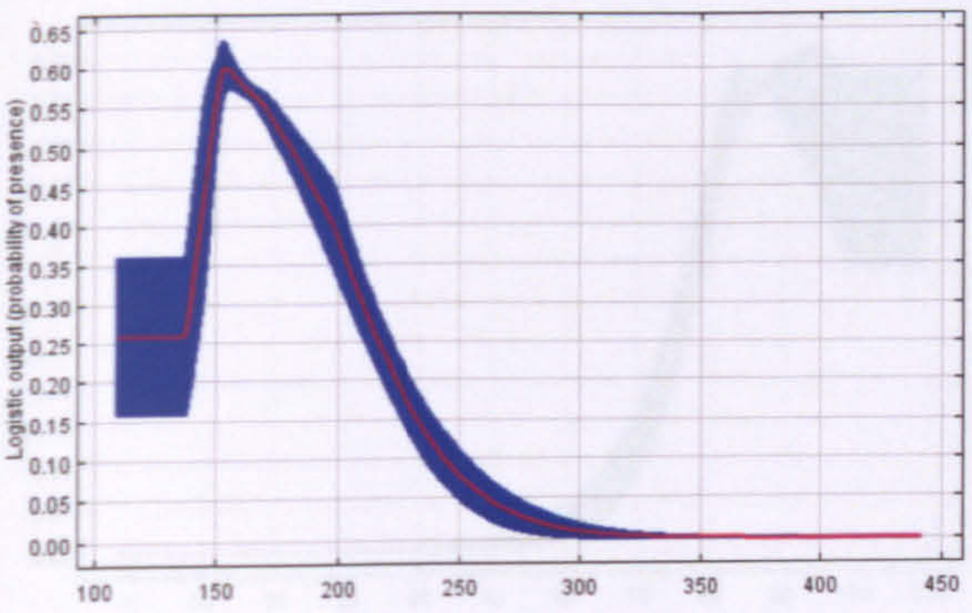
Temperature (non-decimalised °C; i.e. 150 = 15.0 °C)

Geology



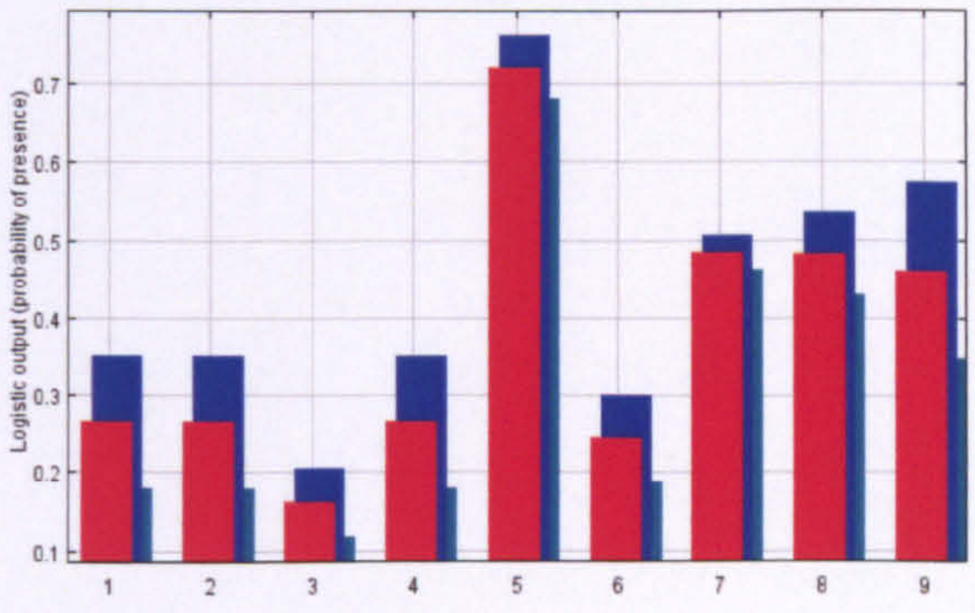
Bedrock class (refer to figure legend below)

Total summer rainfall



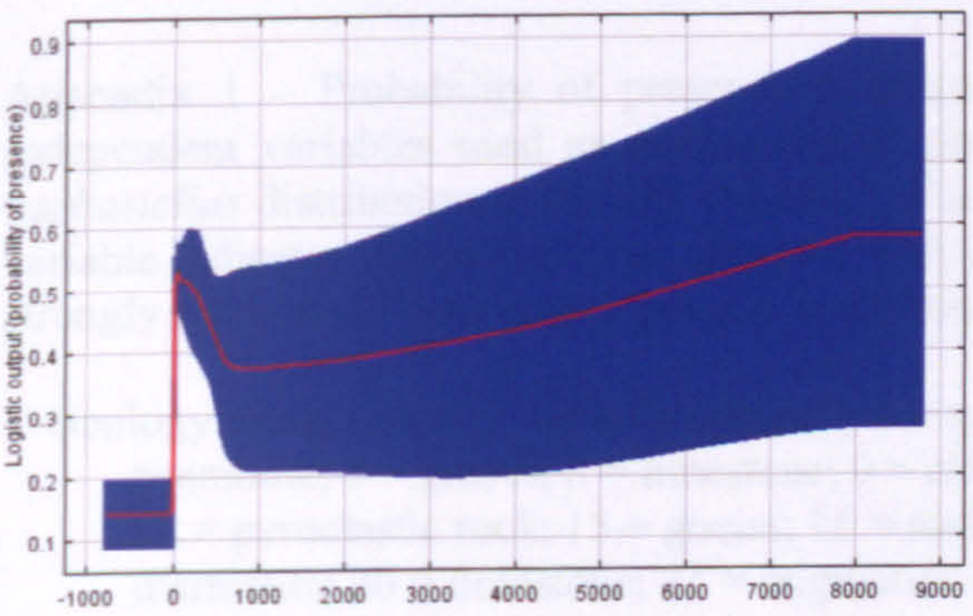
Total rainfall (mm)

Land cover



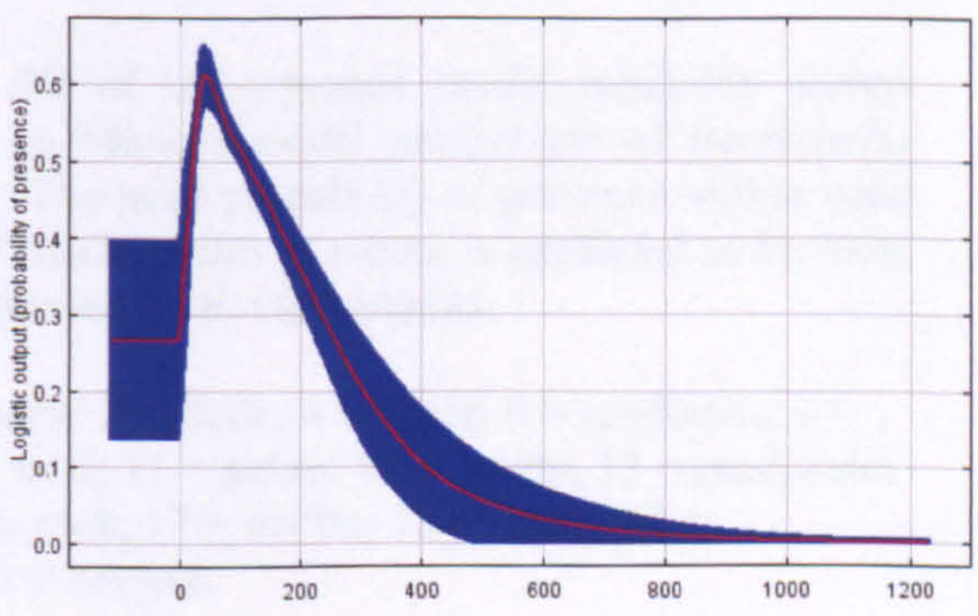
Habitat class (refer to figure legend below)

Human population density



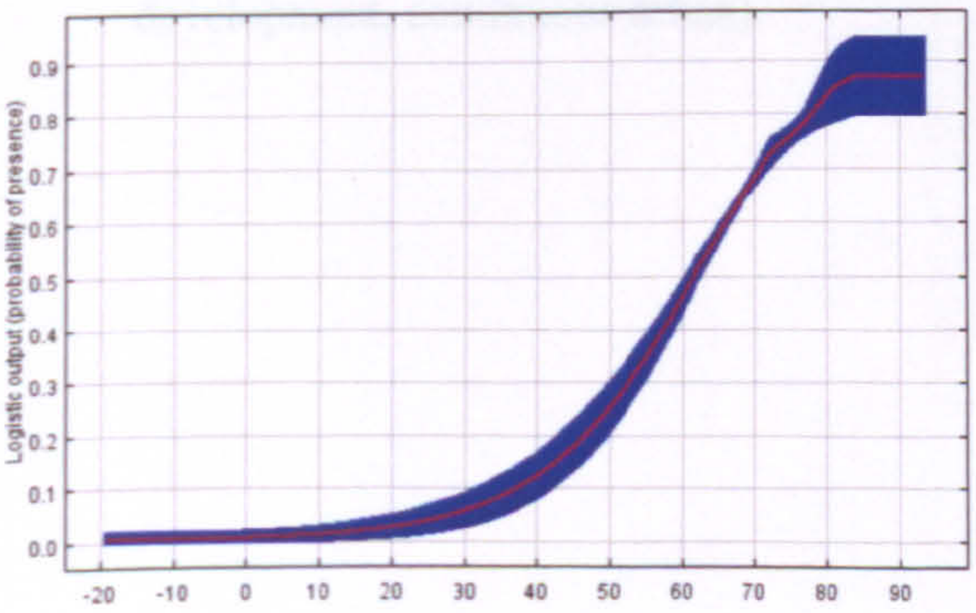
Density (unknown units)

Altitude



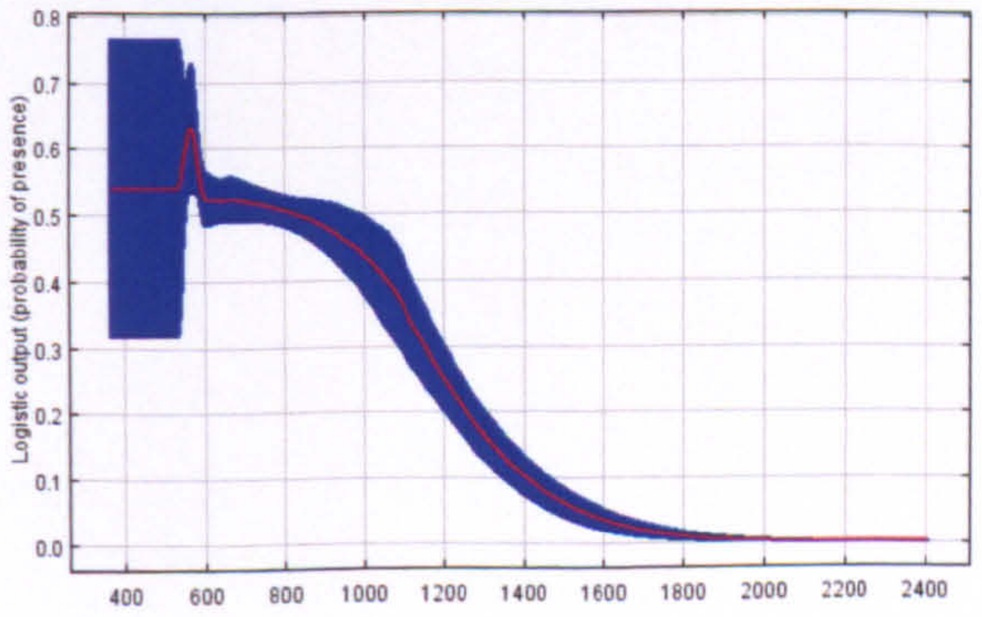
Height above sea level (m)

Maximum January temperature

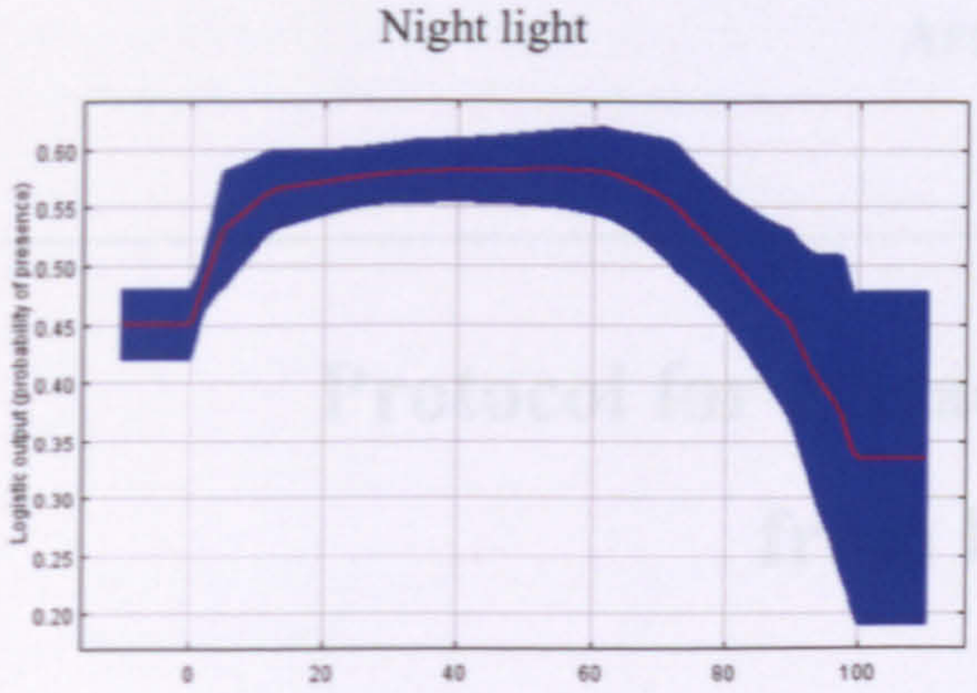


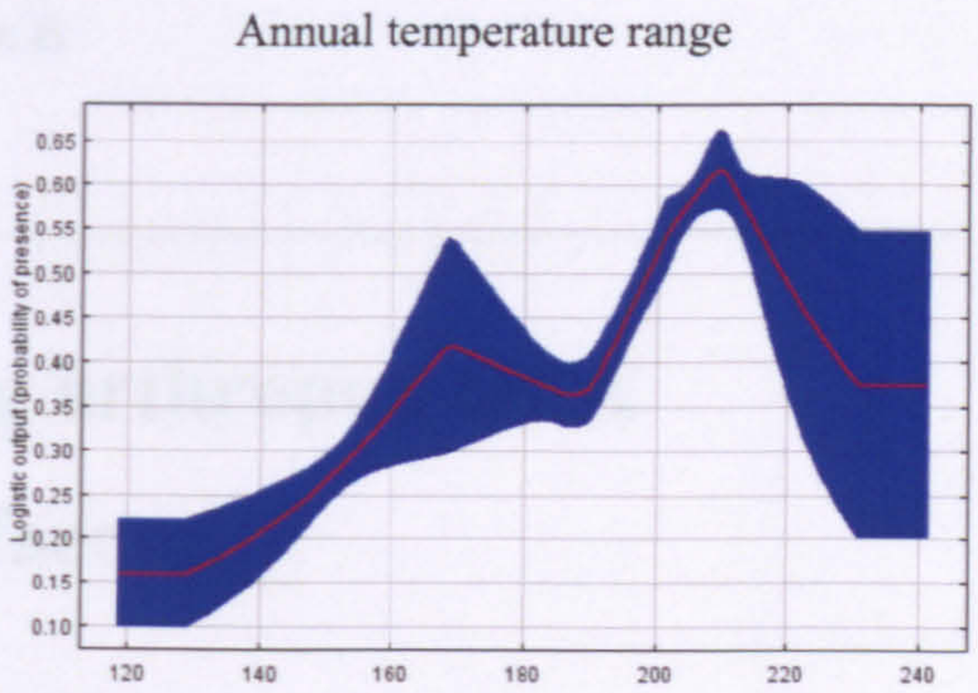
Temperature (non-decimalised °C; i.e. 50 = 5.0 °C)

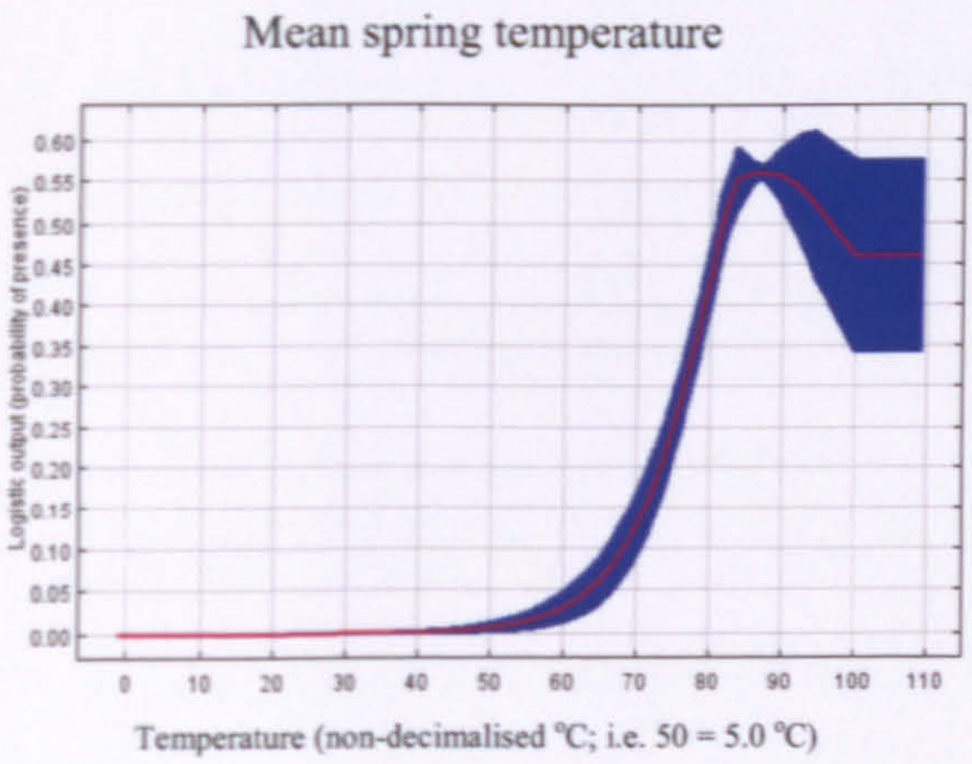
Total annual rainfall



Total rainfall (mm)







Appendix 1 – Probability of presence plots (mean ± *SD* of ten repeated model runs) for eleven independent variables used as environmental predictors in Maxent model predictions of *Barbastella barbastellus* distribution in the UK (Model 1; Chapter 2). The peak probability of presence within each variable indicates the condition or category with which *B. barbastellus* presence is predicted to be most strongly associated. Zero values indicate conditions not tolerated by *B. barbastellus*.

Geology categories: 1 = mafic/lava/basalt; 2 = symetic rock; 3 = chalk; 4 = felsic; 5 = sandstone; 6 = psammite; 7 = gravel; 8 = limestone; 9 = clay; 10 = sand; 11 = pelite; 12 = quartz; 13 = mudstone; 14 = pyroclastic rock; 15 = gneiss; 16 = sedimentary rock; 17 = mafite; 18 = schist; 19 = diamictite; 20 = dolostone; 21 = migmatitic rock; 22 = breccia.

Land cover categories: 1 = water (sea & inland); 2 = rock (littoral, sediment, peat); 3 = shrub heath (includes bracken); 4 = montane habitats; 5 = broadleaved/mixed woodland; 6 = coniferous woodland; 7 = grassland (improved & unimproved); 8 = arable; 9 = urban (suburban, rural development, continuous urban).

Protocol for extracting arthropod DNA from bat faeces

DNA extraction from bat droppings (EtOH stored) using Qiagen QIAamp DNA Stool Kit (Handbook August 2001: adapted protocol for Isolation of DNA from stool for Pathogen Detection)

NB: This protocol is designed to maximise extraction of insect prey DNA from bat faeces. It does not prevent or exclude the extraction of bat, bacterial, fungal, or other non-prey DNA from faecal samples.

NB: The amounts of buffers and reagents given in this protocol are adapted for 10-100mg of faeces (wet weight).

1) Take one faeces from storage and swab excess ethanol with a sterile paper towel and place in a 2ml centrifuge tube.

2) Add 1.4 ml Buffer ASL to the faecal sample. Vortex continuously for 1-3 min or until the stool sample is thoroughly homogenized (nb: a sterile toothpick can be used to aid the break up of the stool).

3) Heat the suspension for 10 min at 70°C in a water bath.

4) Vortex for 30 sec and centrifuge sample at 13,000 rpm for 1 min to pellet faecal particulate.

5) Pipet 1.2 ml of the supernatant into a new 2 ml centrifuge tube (nb: the remaining faecal material can be stored and used for microscopic analysis if required, otherwise discard the pellet).

6) Add 1 InhibitEX tablet to the sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to absorb to the InhibitEX matrix (nb: smaller quantities of tablet should be used for smaller samples).

7) Centrifuge sample at 13,000 rpm for 3 min to pellet inhibitors bound to InhibitEX.

8) Pipet all the supernatant into a new 1.5 ml centrifuge tube and discard the pellet. Centrifuge the sample at full speed for 3 min (nb: transfer of small quantities of pellet material will not affect the procedure).

9) Pipet 20 ul Proteinase K into a new 1.5 ml centrifuge tube, then add 400 µl of supernatant from step 8.

10) Add 400 µl Buffer AL, vortex for 15 sec, and incubate at 70 °C for 15 min (nb: the mixture should be homogenous prior to incubation. Do not add Proteinase K directly to Buffer AL).

11) Add 400 µl of ethanol (96-100%) to the lysate and mix by vortexing. Centrifuge briefly to remove any condensation from the lid of the centrifuge tube.

12) Carefully apply 600µl of the lysate to a QIAmp spin column without moistening the rim. Centrifuge at 13,000 rpm for 1 min. Place spin column in a new collection tube and discard the tube containing the filtrate.

13) Repeat step 12 to load the second aliquot of the lysate to the spin column.

14) Carefully open the spin column and add 500 µl Buffer AW1. Centrifuge at 13,000 rpm for 1 min. Place spin column in a new collection tube and discard the tube containing the filtrate.

15) Carefully open the spin column and add 500 µl Buffer AW2. Centrifuge at 13,000 rpm for 3 min.

16) Place spin column in a new collection tube. Centrifuge at 13,000 rpm for 1 min. Discard tube containing filtrate.

17) Transfer the spin column into a new 1.5 ml centrifuge tube and pipette 50µl Buffer AE directly onto the spin column membrane. Incubate for 1 min at room temperature then centrifuge at 13,000 rpm for 1 min to elute DNA.

Paper arising from this research

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DNA BARCODING

Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces

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Abstract

The application of DNA barcoding to dietary studies allows prey taxa to be identified in the absence of morphological evidence and permits a greater resolution of prey identity than is possible through direct examination of faecal material. For insectivorous bats, which typically eat a great diversity of prey and which chew and digest their prey thoroughly, DNA-based approaches to diet analysis may provide the only means of assessing the range and diversity of prey within faeces. Here, we investigated the effectiveness of DNA barcoding in determining the diets of bat species that specialize in eating different taxa of arthropod prey. We designed and tested a novel taxon-specific primer set and examined the performance of short barcode sequences in resolving prey species. We recovered prey DNA from all faecal samples and subsequent cloning and sequencing of PCR products, followed by a comparison of sequences to a reference database, provided species-level identifications for 149/207 (72%) clones. We detected a phylogenetically broad range of prey while completely avoiding detection of nontarget groups. In total, 37 unique prey taxa were identified from 15 faecal samples. A comparison of DNA data with parallel morphological analyses revealed a close correlation between the two methods. However, the sensitivity and taxonomic resolution of the DNA method were far superior. The methodology developed here provides new opportunities for the study of bat diets and will be of great benefit to the conservation of these ecologically important predators.

Keywords: Chiroptera, COI, diet analysis, DNA barcoding, species identification, taxon-specific primers

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Introduction

Determining the dietary habits of bats is central to understanding their trophic relationships within ecosystems and is a key part of their conservation management. Because direct observations of feeding events are often impossible, diets of insectivorous bats are studied conventionally by morphological identification of microscopic prey remains, primarily fragments of arthropod cuticle, which remain in faeces (e.g. Beck 1995; Rydell *et al.* 1996; Sierro & Arlettaz 1997). However, the thorough mastication and digestion of prey by bats coupled with low morphological disparity among related arthropods restricts most practicable taxonomic identifications to order and hence offers only a limited perspective on diet.

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Molecular techniques provide alternative approaches to the study of animal diets. Of those described, DNA-based approaches are perhaps the most suitable for examining the range and diversity of prey taken by generalist predators (Symondson 2002). Through polymerase chain reaction (PCR) amplification of DNA sequences unique to prey species, identifications can be achieved even within highly degraded samples such as those found in faeces, gut contents or regurgitates (King *et al.* 2008). This approach has recently been applied to a range of predator groups, including: marine vertebrates (Jarman *et al.* 2002; Jarman & Wilson 2004; Deagle *et al.* 2005a,b; Parsons *et al.* 2005); seabirds (Deagle *et al.* 2007); marine invertebrates (Blankenship & Yayanos 2005; Braley *et al.* 2009); insectivores (Clare *et al.* 2009); terrestrial invertebrates (Hoogendoorn & Heimpel 2001; Pons 2006; Garros *et al.* 2008); herbivores (Pegard *et al.* 2009; Soininen *et al.* 2009); and to broader studies of trophic ecology (e.g. Carreon-Martinez & Heath 2010; Corse *et al.* 2010).

The successful identification of anonymous DNA sequences to species relies on two important conditions being met. First, sequence divergence at genetic markers must be sufficient to deliver species resolution, and second, reference sequences of the same species are required to ensure that an accurate sequence diagnosis can be made. Recent DNA-based diet studies have therefore targeted DNA barcoding regions to achieve high taxonomic resolution (Hebert *et al.* 2003a,b) and to make use of rapidly developing 'barcode' libraries [Barcode of Life Database (BOLD), Ratnasingham & Hebert (2007)]. In degraded faecal samples, however, the propensity for DNA sequences >300 base pairs (bp) to survive digestion can be very low, inhibiting the recovery of full COI barcodes (a 658- bp region of the mitochondrial cytochrome *c* oxidase I (COI) gene) (Deagle *et al.* 2006). Recent examination of 'mini-barcodes' suggests that even very short fragments (100–250 bp) of the complete COI barcode region can deliver 90-95% species-level resolution and are easily recovered from degraded samples (Hajibabaei *et al.* 2006; Meusnier *et al.* 2008). Hence, DNA barcoding using mini-barcode markers can provide an effective solution to obtaining prey species identifications from animal faeces where morphological assessment of prey hard-parts is problematic.

The diversity of prey available to aerial insectivores is considerable. Even bats with 'specialized' diets may consume hundreds of different species. Despite previous publication of numerous arthropod primer sets, to the best of our knowledge, none developed thus far can perform as taxon-specific primers for COI barcoding arthropod prey in the faeces of vertebrate predators. In the only DNA-based study of bat diets to date, Clare *et al.* (2009) relied on existing COI barcode primers (LepF1/LepR1, Hebert *et al.* (2004)) to recover DNA sequences from prey fragments individually isolated from faeces. While successful in detecting a large variety of prey species, nontarget templates were also amplified, including bacterial, fungal and bat DNA. Although only 3% of sequences were derived from bats, the authors noted that other bat species with stronger sequence similarity to primers may cause significant interference, necessitating the development of new primer sets.

In this study, we aimed to develop and validate a universal PCR-based methodology for the study of insectivorous bat diets. For this purpose, we targeted very short mtDNA barcode fragments that are expected to remain in degraded faecal samples yet still possess sufficient sequence information to provide species resolution of prey items. More specifically, our objectives were: (i) to design a novel taxon-specific primer set for the universal amplification of arthropod COI mini-barcodes; (ii) to examine nucleotide-sequence divergence at the corresponding mini-barcode marker to test performance in providing species-level diagnoses; (iii) to demonstrate a complete working methodology via empirical tests using faecal samples obtained from three diet-differentiated bat species; and (iv) to examine the potential for making DNA-based assessments of diet composition by comparing information obtained through conventional and DNA-based diet analyses.

Materials and methods

Primer design

Primers were designed using COI barcode sequences obtained from GenBank for 11 arthropod orders (10 from Insecta; one from Arachnida) found in the diets of insectivorous bats (Vaughan 1997) and a range of nontarget taxa, including bat, bacteria and fungi, which may also be represented within faecal samples (Clare *et al.* 2009). Sequences were aligned in BioEdit (Hall 1999) using ClustalW (Thompson *et al.* 1994), and, where available, arthropod orders were represented by >2 families. Regions of DNA that were conserved among arthropods and had low similarity in nontarget taxa were identified as sites for potential primer synthesis. Primers were designed for a number of appropriate binding sites within the full 658- bp barcode region, and primer combinations expected to amplify 100–300 bp fragments were tested empirically for specificity using DNA templates purified from a range of target and nontarget specimens. After initial screening of unsuccessful primer combinations (i.e. those that amplified from nontarget taxa or did not universally amplify arthropod taxa), a single primer set exhibiting the required 'taxon-specific' qualities (Table 1) was selected for PCR amplification of arthropod mini-barcodes (157 bp) from faecal samples.

Analysis of marker performance

COI barcodes for all available species in the class Insecta were downloaded from BOLD and trimmed to match the 157 -bp marker region to provide a data set from which examinations of nucleotide-sequence divergence could be made. After removal of sequences that were incomplete or lacked species labels, a final data set of 38 603 sequences representing 6867 species, 2669 genera, 260 families and 23 orders was used to calculate divergence values at each corresponding taxonomic level. For analysis at order level, two orders were selected randomly from the data set, and for each of these, an example species was chosen at random. The sequences from these two species were compared, and their sequence divergence score calculated as the number of differing bases divided by the aligned sequence length (*p*-distance,

Table 1 Taxon-specific PCR primer set developed for this study. Orders successfully amplified by the primer set (Primer specificity: ✔) are shown alongside the relative frequencies with which they occur in Chiropteran diets (✔✔✔ frequent, ✔ occasional or rare; Vaughan 1997). The length of the amplified fragment is 157 bp

Name	Sequence 5′ to 3′
ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG
ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC

Class: Order	Dietary composition	Primer specificity
Insecta		
Ephemeroptera	✔	✔
Odonata	✔	✔
Plecoptera	✔	✔
Orthoptera	✔	✔
Dermaptera	✔	✔
Hemiptera	✔✔✔	✔
Neuroptera	✔✔✔	✔
Lepidoptera	✔✔✔	✔
Trichoptera	✔✔✔	✔
Diptera	✔✔✔	✔
Hymenoptera	✔✔✔	✔
Coleoptera	✔✔✔	✔
Arachnida		
Araneae	✔	✔
Mammalia		
Chiroptera	×	×

Hebert *et al.* 2003a). This sampling was repeated 1000 times in total, choosing two orders at random in each case. The mean, standard deviation and 95% confidence intervals were calculated from the resulting sample of 1000 divergence scores. This entire sampling process was then repeated at the family, genus and species levels. For within-species analysis, the same process was applied to comparisons of conspecific sequence pairs. All divergence calculations were performed using a PERL script (PERL 5.8.8 http://www.perl.org). To measure the overall resolution of mini-barcodes, sequences from all species present in the data set were compared and the proportion of nonoverlapping barcodes (i.e. barcodes that uniquely identified species) was determined.

Collection of dietary samples

Faeces were collected from 15 bats (11 *Barbastella barbastellus*; 2 *Pipistrellus pipistrellus*; 2 *Myotis nattereri*) caught under licence using mist nets and harp traps within woodlands in southern England. These bat species exploit different dietary niches and collectively prey upon a broad range of arthropod taxa (Vaughan 1997). Sampling from these species therefore provided an opportunity to test primer performance in detecting a phylogenetically diverse group of prey from a variety of DNA matrices. Individual bats were held in sterilized holding bags for a maximum of 30 min or until they defecated, after which time they were released. Any resulting faeces were immediately stored in 100% ethanol to preserve DNA samples prior to analysis.

DNA extraction and PCR amplification

For each bat, DNA was extracted from a single faecal pellet weighing 10–50 mg (average: 27 mg) with the DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions with modifications (see supplementary information Data S1). Negative control extractions were performed alongside each batch of extractions from faecal samples to monitor for contamination. All PCRs were carried out in 10 μL volume reactions using the BIOTAQ PCR kit (Bioline). Each PCR contained 1 μL 10 × NH4 buffer, 4.55 μL deionized water, 0.4 μL 50 mM $MgCl_2$ solution, 1 μL 2 mM dNTPs, 1 μL forward primer, 1 μL reverse primer, 0.05 μL BIOTAQ DNA polymerase and 1 μL of DNA template from the final extraction elutions. A touch-down PCR protocol was used to incorporate annealing temperatures of forward (ZBJ-ArtF1c: 56.4 °C) and reverse (ZBJ-ArtR2c: 57.7 °C) primers. The PCR thermal cycling conditions were as follows: 3 min at 94 °C followed by 16 cycles of 30 s at 94 °C, 30 s at 61 °C (decreased by 0.5 °C per cycle) and 30 s at 72 °C followed in turn by 24 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C followed by a final incubation of 10 min at 72 °C. PCR products were visualized on a 1.5% agarose gel, and remaining PCR volumes were purified using the QIAquick PCR Purification Kit (Qiagen). Final elution volumes were adjusted for each sample to optimize DNA concentrations for cloning.

Clone library construction and sequencing

PCR products were cloned using the pGEM-T Easy Vector System and high-efficiency competent cells (≥1 × 10^8 cfu μg^{-1} DNA) (Promega). Optimal recombinations were achieved using a 1:1 insert to vector molar ratio and when ligation reactions were incubated overnight at 4 °C. Competent cells were transformed by applying 1 μL of ligation product to 50 μL cells and heat-shocked as follows: 30 min on ice, 25 s at 42 °C (water bath) and 2 min on ice. Colonies containing recombinant clones were selected by X-gal-mediated blue/white selection and cultured in 50 μL LB broth at 37 °C for 16–18 h. Cloned inserts were liberated from cells and amplified via PCR using M13 primers. The resulting amplicons were bi-directionally sequenced using BigDye (version 3.1) chemistry and an ABI3730xl automated sequencer (Applied Biosystems Incorporated).

Identification of DNA sequences

Sequence data were compiled in BioEdit and aligned using ClustalW. Vector and primer sequences were removed, and forward and reverse sequences from each clone were compared to identify sequencing error. Sequences were examined further for unexpected insertions/deletions, frameshift mutations and in-frame stop codons to screen for nuclear mitochondrial pseudogenes (numts), which can be co-amplified with mitochondrial DNA (mtDNA) paralogues and lead to false interpretations of diet composition (Dunshea *et al.* 2008; Song *et al.* 2008; Moulton *et al.* 2009). Other potentially spurious sequences were identified by aligning sequences to authentic mtDNA obtained from BOLD and examining where substitutions occurred in 'suspect' sequences in relation to phylogenetically conserved positions (Dunshea *et al.* 2008). After screening away suspect sequences, all remaining sequences were compared to reference sequences in BOLD to obtain 'closest match' identifications. Identifications to order, family, genus or species were made according to per cent similarity of sequence matches. The lower 95% CI for marker divergence at each level of taxonomic affinity were chosen as conservative 'cut-offs' to control for false-positive identifications (type I error) (Table 2). Accordingly, identifications to order, family, genus or species were made when sequence similarities exceeded 85.9%, 91.0%, 94.9% and 99.3% respectively. Sequences with equal similarity to two or more taxa were identified to the higher taxonomic level that included both taxa.

Morphological vs. DNA-based analysis

DNA extractions from faeces were designed such that all solid faecal material could be retained for additional morphological analysis of hard-parts. Initial examination showed faecal material was not noticeably degraded by extraction processes and an analysis of prey hard-parts could be performed following conventional methods (Whitaker *et al.* 2009). Hard-part fragments were examined under a binocular microscope and compared to reference fragments from vouchered arthropod specimens to allow identification of prey to order. For *B. barbastellus* ($n = 11$), per cent frequency of occurrence (%FO, proportion of samples containing a given prey taxon) and per cent volume (%V, volume of a given prey taxon expressed as a proportion of the total diet) were used to quantify morphological data (Whitaker *et al.* 2009), and %FO and per cent clones (proportion of sampled clones containing DNA from a given prey taxon) – which have previously been shown to correspond roughly with known dietary proportions (Deagle *et al.* 2005b) – were used to quantify DNA data. For *Myotis nattereri* and *Pipistrellus pipistrellus* (both $n = 2$), the comparison of morphological and DNA-based diet data was based on presence/absence of prey taxa only. To investigate whether Lepidoptera, Coleoptera and Diptera (the major food of insectivorous bats) could be detected equally within faeces, we fed a single captive brown long-eared bat (*Plecotus auritus*) a series of five treatment meals (comprising waxworms *Galleria mellonella* (Lepidoptera larvae), mealworms *Tenebrio molitor* (Coleoptera larvae) and casters *Calliphora vomitoria* (Diptera pupae)) over a period of 15 days and analysed subsequent faecal samples using both DNA and morphological methods.

Results

Marker performance

Mean divergences at order, family, genus and species level were 22.5%, 20.0%, 13.6% and 7.0%, respectively (Table 2). At order and family level, all 1000 species pairs could be resolved. At genus level, one species pair (0.1%) could not be resolved, and at species level, 22 pairs (2.2%) were unresolved. When all 6867 species in the data set were compared, 6617 possessed unique mini-barcodes, providing an overall species resolution of 96.4%. Within species, 25.4% of conspecific pairs showed

Table 2 Divergence values calculated for each taxonomic level within the class Insecta. *n* is the number of different taxa at each level of taxonomic affinity from which example species (or individuals for within-species) were randomly selected, and *n reps* is the number of randomly sampled species (or individuals) pairs that were compared to calculate divergence values. Unresolved branches are the number of sequence pairs with nonunique mini-barcodes (zero divergence)

Taxonomic level	*n*	Mean	SD	Upper 95% CI	Lower 95% CI	*n* reps	Unresolved branches
Order	23	22.5	5.2	32.5	14.1	1000	
Family	260	20.0	6.8	37.0	9.0	1000	
Genus	2669	13.6	5.0	24.9	5.1	1000	1
Species	6867	7.0	4.4	17.2	0.7	1000	22
Within-species	38603	0.5	1.5	3.2	0.0	1000	746

sequence divergence, and mean divergence for all 1000 pairs was 0.5%.

DNA-based identification of prey

All faecal samples yielded amplifiable DNA and PCRs produced successful amplifications on all attempts. All PCR amplifications produced single bands, and cloning of PCR products typically yielded more than 50 recombinant clones per library. DNA sequencing of 240 clones (16 per faecal sample) produced 215 readable sequences, and examination of these sequences revealed no indels, frameshift mutations or in-frame stop codons. Eight sequences showed single base substitutions in phylogenetically conserved positions and were ear-marked as suspected spurious sequences for removal from the data set. Further examination revealed seven of these 'spurious' sequences matched to prey species that had already been identified within faecal samples. Furthermore, each 'spurious' sequence was only detected once within the complete data set. Consequently, their removal from the data set had little effect on the overall assessment of diet. Of the remaining 207 sequences, 72% showed >99.3% similarity to reference sequences on BOLD and were identified to species (or to genus in cases of equal similarity to >1 reference sequence). All other sequences except one were at least 95% similar to reference sequences and were therefore identified to genus. The final sequence (94.9% similarity) was identified to family. Overall, 37 different prey taxa were identified from the 15 faecal samples analysed (Table 3). Thirty-six of these, represented by 206 sequences (99.5%), belonged to the class Insecta. The final sequence was derived from a spider (Arachnida: Araneae). Crucially, no nontarget taxa were detected. All haplotype prey sequences have been deposited in the European Nucleotide Archive (ENA), under accession numbers FR682940–FR682997.

Morphological vs. DNA-based analysis

Of the 15 faecal samples examined, morphological analysis identified prey remains from three distinct insect orders; Lepidoptera, Diptera and Neuroptera. In contrast, seven orders were identified by DNA-based methods (Table 4). For all three bat species, prey orders were identified in DNA analyses that were otherwise missed in morphological analyses. For *B. barbastellus*, Lepidoptera was the most dominant prey group. This was evident from both morphological (mean %FO: 100; mean %V: 96) and DNA-based analyses (mean %FO: 100; per cent clones (n = 159): 86). Overall, the two diet analysis methods showed a high level of congruency for estimates of diet composition (Table 4). Results from the feeding trial confirmed that when faeces contain the remains of lepidopteran, coleopteran and dipteran prey, the primer set is capable of co-amplifying DNA from all prey types (Table 5).

Discussion

We successfully amplified DNA from the faeces of three insectivorous bat species, supporting previous evidence that DNA derived from arthropod prey regularly survives digestion and can be readily detected via PCR (Clare *et al.* 2009). Moreover, by targeting a short but informative COI barcode marker, we were able to detect a broad range of prey and make species-level identifications within all faecal samples. Our diagnoses of prey species are likely to be highly robust as, in contrast to other diet studies that have used arbitrary per cent similarity criteria to accept species-level identifications, the parameters used for taxonomic assignment in this study were derived directly from calculations of marker divergence among prey taxa. Given that DNA derived from prey taxa may represent only a small fraction of the total DNA in predator faeces (Deagle *et al.* 2006; Vestheim & Jarman 2008) and that bats are readily identified from their own faeces via PCR (Vege & McCracken 2001; Puechmaille *et al.* 2007), our success in completely avoiding detection of nontarget taxa was significant. Furthermore, we were able to amplify DNA from spiders, a phylogenetic outlier among insectivorous bat prey, suggesting that this primer set is capable of detecting the complete range of arthropod prey in diets.

Despite being one quarter the size, the mini-barcode marker targeted by our primer set showed similar performance in resolving prey taxa to that expected of full-length barcodes. On only three occasions did we find that prey items could not be resolved at the species level, despite having a > 99.3% similarity score. In each case, the prey sequence matched with equal similarity to two congeneric lepidopteran species, so identifications to genus were made instead. Among all the insects, the lepidopterans pose a particularly challenging case for species diagnosis; they are one of the most taxonomically diverse orders and show lower than average divergence among congeneric species (Hebert *et al.* 2003a,b). Given that we were able to identify the large majority of lepidopteran prey to species, we expect that other prey groups would be equally, if not more highly, resolved.

Although we found no clear evidence of mitochondrial pseudogenes among prey sequences, numts may be common among insect taxa (Moulton *et al.* 2009) and some numts lack characteristic mutations (including indels, frame-shift mutations and in-frame stop codons) and can be difficult to differentiate from their mitochondrial paralogues (Song *et al.* 2008). Therefore, we cannot completely rule out the possibility of numt

Table 3 List of prey identified in the faeces of 11 *Barbastella barbastellus*, 2 *Myotis nattereri* and 2 *Pipistrellus pipistrellus* by DNA analysis, showing per cent similarity of closest matches to reference sequences on BOLD. 'Unknown' identifications are provided where similarity scores were not sufficient to permit identification or where reference sequences lacked taxon labels

Order	Family	Genus	Species ID	% similarity
B. barbastellus				
Araneae	Tetragnathidae	*Metellina*	*Metellina segmentata*	100.0
Diptera	Drosophilidae	*Drosophila*	*Drosophila* sp.	98.7
	Scathophagidae	*Scathophaga*	*Scathophaga stercoraria*	99.4
	Tipulidae	Unknown	Unknown sp.	97.4
Lepidoptera	Arctiidae	*Spilosoma*	*Spilosoma lubricipeda*	100.0
			Spilosoma luteum	100.0
	Geometridae	*Cyclophora*	*Cyclophora punctaria*	100.0
		Ennomos	*Ennomos quercinaria*	100.0
		Odontopera	*Odontopera bidentata*	100.0
		Petrophora	*Petrophora chlorosata*	100.0
	Incurvariidae	*Nematopogon*	*Nematopogon swammerdamella*	99.4
			Nematopogon sp.	98.7
	Lymantriidae	*Calliteara*	*Calliteara pudibunda*	100.0
	Noctuidae	*Agrotis*	*Agrotis exclamationis*	100.0
		Apamea	*Apamea monoglypha*	100.0
		Conistra	*Conistra* sp.	100.0
		Diarsia	*Diarsia* sp.	100.0
		Hoplodrina	*Hoplodrina ambigua*	100.0
		Noctua	*Noctua pronuba*	100.0
			Noctua sp.	100.0
		Ochropleura	*Ochropleura plecta*	100.0
		Orthosia	*Orthosia cerasi*	100.0
		Phlogophora	*Phlogophora meticulosa*	100.0
	Pyralidae	*Plodia*	*Plodia interpunctella*	100.0
Neuroptera	Chrysopidae	Unknown	Unknown sp.	95.7
M. nattereri				
Coleoptera	Carabidae	Unknown	Unknown sp.	96.4
Diptera	Anthomyiidae	*Delia*	*Delia* sp.	99.4
	Chloropidae	Unknown	Unknown sp.	97.9
	Empididae	Unknown	Unknown sp.	96.8
	Syrphidae	*Melanostoma*	*Melanostoma scalare*	100.0
Plecoptera	Pteronarcyidae	Unknown	Unknown sp.	96.3
P. pipistrellus				
Diptera	Tachinidae	Unknown	Unknown sp.	96.4
	Limoniidae	Unknown	Unknown sp.	100.0
Ephemeroptera	Heptageniidae	*Epeorus*	*Epeorus* sp.	97.4
Lepidoptera	Tortricidae	Unknown	Unknown sp.	94.9
	Gracillariidae	*Cameraria*	*Cameraria ohridella*	100.0
	Incurvariidae	*Incurvaria*	*Incurvaria masculella*	100.0

co-amplification in this study. The eight sequences we identified as 'spurious' were unlikely numt candidates as in each case they differed by only one base from putative mitochondrial orthologues recovered from the same faeces [numts typically show greater divergence from mitochondrial paralogues (Bensasson *et al.* 2001)]. We suspect instead that these anomalous sequences are products of PCR error. Although such errors are largely unavoidable, within any one 157-bp amplicon, the probability of incurring a sequence error exceeding one base (0.6%) is low. Consequently, the majority of sequences will still be correctly identified, as single base divergences are a closer reflection of that found among conspecifics (mean: 0.5%) than among congenerics (mean: 7.0%). Indeed, this appears to be true for seven of the eight 'spurious' sequences identified in this study.

Genetic material within faeces is invariably heavily degraded, and the recovery of sequences larger than 300 bp may be extremely difficult, if not impossible (Deagle *et al.* 2006). This is likely to be true of insectivorous bats, which chew and digest their prey thoroughly. By targeting a short (157 bp) multi-copy mtDNA marker, we were able to amplify prey templates from all faeces that we tested, irrespective of sample quality. Previous

Table 4 Diet composition of three bat species (*B. barbastellus*, $n = 11$; *Myotis nattereri*, $n = 2$; *Pipistrellus pipistrellus*, $n = 2$) using calculations of per cent frequency of occurrence (%FO), per cent volume (%V), per cent clones, and presence (Y) data for morphological and DNA-based assessments of arthropod prey in faeces

	B. barbastellus				*M. nattereri*		*P. pipistrellus*	
	%FO		# (%) clones	%V				
Prey order	DNA	Morph	DNA	Morph	DNA	Morph	DNA	Morph
Lepidoptera	100	100	137 (86)	96			Y	Y
Diptera	27	27	16 (10)	3	Y	Y	Y	Y
Neuroptera	9	9	5 (3)	1				
Araneae	9		1 (1)					
Coleoptera					Y			
Plecoptera					Y			
Ephemeroptera							Y	

Table 5 DNA and morphological detection of prey in faecal samples collected from a single brown long-eared bat (*Plecotus auritus*) during a controlled feeding trial. Starvation periods ensured complete consumption of meals when offered. Meals included 'mealworm' (larvae of the Mealworm beetle, *Tenebrio molitor*); 'caster' (pupae of the Blue bottle fly, *Calliphora vomitoria*); 'waxworm' (larvae of the Wax moth, *Galleria mellonella*); and 'mixed' (equal parts by volume of each food type)

				DNA (number (%) of clones)			Morphological (per cent volume)		
Day	Treatment	Meal	Sample† (after meal)	Mealworm	Caster	Waxworm	Mealworm	Caster	Waxworm
1	1	mealworm	10 h	3 (100)			100		
2		mealworm	22 h	2 (100)			100		
3	Starve								
4	2	mixed	20 h	9 (64)		5 (36)	50	30	20
5		mixed	9 h	8 (50)	3 (19)	5 (31)	40	30	30
6	Starve								
7	3	mixed	24 h	9 (64)	3 (21)	2 (14)	50	40	10
8		mixed	6 h	9 (64)	5 (36)		40	40	20
9	Starve								
10									
11	4	caster	23 h		3 (100)			100	
12		caster	9 h		2 (100)			100	
13	Starve								
14	5	waxworm	22 h		3 (100)			60	40
15		waxworm	7 h			3 (100)			100

†Time of faecal collection given as the number of hours after each meal was offered.

studies have noted prey-specific biases in DNA survival during digestion (e.g. Deagle & Tollit 2007). If this is true for bats, it would be reasonable to predict that larger, hard-bodied prey, which are disproportionately well represented in solid faecal material, would be preferentially detected. Our detection of small, soft-bodied micro-moths among larger, more heavily sclerotized prey suggests that, if a bias in DNA survival does occur, this method still provides adequate sensitivity to detect those prey items most vulnerable to digestion.

The three bat species sampled in this study occupy distinct dietary niches. *B. barbastellus* specializes in eating lepidopteran moths taken by aerial hawking; *P. pipistrellus* takes mainly aquatic Diptera, also by hawking; and *M. nattereri* takes almost entirely diurnal Diptera gleaned from their nightly resting places. Typically, however, the diets of all these species contain a broad range of prey orders (reviewed in Vaughan 1997). The clone libraries generated for each of these species closely reflect previous descriptions of diet. For *B. barbastellus*, 137/159 clones (86%) were matched to 20 different moth species, while 16 clones (10%) were identified as dipteran; for *M. nattereri*, 29/31 clones (94%) were identified as dipteran; and for *P. pipistrellus*, 10/25 clones (40%) were matched to dipterans, while 12 clones (48%) were identified to three different micro-moths. All of the prey items

we identified are typical of what might be encountered by these bat species, suggesting that our analysis appears not to have been confounded by interference from secondary predation (Sheppard *et al.* 2005).

A comparison of DNA data with those obtained via morphological analysis shows that a greater diversity of prey was detected via DNA-based analyses. Currently, DNA-based approaches to diet analysis provide only limited scope for making quantitative interpretations of diet composition (King *et al.* 2008). The use of clone libraries to estimate prey proportions represents one possible interpretation that has previously been shown to correspond roughly with known dietary proportions (Deagle *et al.* 2005b). Our own assessment of clone libraries for *B. barbastellus* provided an estimate of diet composition that closely resembled that calculated from morphological data, both of which corresponded well with previous diet studies for this species (Rydell *et al.* 1996; Sierro & Arlettaz 1997). Whether the same level of congruence can be achieved for larger sample sizes or for different bat species with more complicated diets remains to be seen. Meanwhile, other techniques such as high-throughput pyrosequencing are also proving well suited to dietary analysis and may provide more useful interpretations of diet composition (Deagle *et al.* 2009; Soininen *et al.* 2009; Valentini *et al.* 2009). However, in the absence of a robust method for quantifying faecal DNA, a combination of both DNA-based and conventional techniques is likely to prove most beneficial (Casper *et al.* 2007; Braley *et al.* 2009).

Our success in resolving nearly all prey items to the genus or species level is testament to the role DNA barcoding can play in significantly improving our understanding of animal diets. Barcode libraries are of course an integral part of the equation, and current campaigns to further develop these resources will greatly benefit future diet studies. In summary, this PCR-based method provides an efficient noninvasive tool for providing robust prey species identifications in the diets of insectivorous bats. Moreover, this method may be universally applicable across a broad spectrum of vertebrate insectivores. For insectivorous bats, DNA barcoding of faeces currently provides the only realistic means of determining the range and diversity of prey within diets. As such, this method offers new perspectives and opportunities in the study of bat dietary ecology and predator–prey interactions and may also be significant for the future conservation of bats.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Data S1 DNA extraction from bat droppings (EtOH stored) using Qiagen QIAamp DNA Stool Kit.

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Paper arising from this research

Goerlitz HR, ter Hofstede HM, Zeale MRK, Jones G, Holderied MW

An aerial-hawking bat uses stealth echolocation to counter moth hearing

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Report

An Aerial-Hawking Bat Uses Stealth Echolocation to Counter Moth Hearing

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Summary

Ears evolved in many nocturnal insects, including some moths, to detect bat echolocation calls and evade capture [1, 2]. Although there is evidence that some bats emit echolocation calls that are inconspicuous to eared moths, it is difficult to determine whether this was an adaptation to moth hearing or originally evolved for a different purpose [2, 3]. Aerial-hawking bats generally emit high-amplitude echolocation calls to maximize detection range [4, 5]. Here we present the first example of an echolocation counterstrategy to overcome prey hearing at the cost of reduced detection distance. We combined comparative bat flight-path tracking and moth neurophysiology with fecal DNA analysis to show that the barbastelle, *Barbastella barbastellus*, emits calls that are 10 to 100 times lower in amplitude than those of other aerial-hawking bats, remains undetected by moths until close, and captures mainly eared moths. Model calculations demonstrate that only bats emitting such low-amplitude calls hear moth echoes before their calls are conspicuous to moths. This stealth echolocation allows the barbastelle to exploit food resources that are difficult to catch for other aerial-hawking bats emitting calls of greater amplitude.

Results and Discussion

Aerial-hawking bats pinpoint their airborne insect prey with echolocation calls that are typically among the most intense biological sounds [4, 5]. Ears evolved in many insect taxa together with evasive flight as antipredator adaptations [1]. It is debatable, however, whether bats have coevolved counteradaptations against eared prey [2]. In general, examples of predators prevailing over their prey in the coevolutionary arms race, such as toxin resistance in garter snakes [6], are rare because of lower selection pressure on the predator than the prey (the life/dinner-principle [7]). For bats and their insect prey, the allotonic frequency hypothesis proposes that some bat species responded to hearing prey by calling at frequencies outside the range of the prey's greatest auditory sensitivity [8]. Despite much support for this hypothesis [2, 8], other benefits could have initially driven selection for these changes, such as increased detection distance at low frequencies or improved spatial resolution at high frequencies [3]. Here we present evidence for a previously unknown counteradaptation in the aerial-hawking bat, *Barbastella barbastellus*: the use of low-amplitude calls. This is likely to be a specific adaptation in response to insect hearing because it imposes the cost of reduced prey detection distance to the bat with no compensating benefit other than making its calls inconspicuous to eared prey. There is no energetic benefit to low-amplitude calls in bats [9]; *B. barbastellus* is not known to take prey from surfaces or forage within dense foliage, which would favor low-amplitude calls [10]; and there is no evidence of kleptoparasitism in foraging bats, only by bats that occasionally catch a moth missed by a previous bat [11]. Many gleaning bats (those that take prey from surfaces) also produce low-amplitude calls, but again there are alternative benefits for doing so other than remaining inconspicuous to prey [12]. We compared the diet, detection distances by moths, and call source levels of *B. barbastellus* with those of a similar sympatric aerial-hawking bat species, *Nyctalus leisleri*. Both are medium-sized bats that forage on insects in edge and open habitats [10]. They both call at relatively low ultrasonic frequencies (33 and 28 kHz peak frequency, respectively) that are within the best hearing range of most moth species [8, 13].

B. barbastellus preys almost exclusively on moths (reviewed in [14]), but not all moths have ears. Conventional diet studies have identified prey remains within feces by using microscopic techniques, which provide only limited resolution of prey (typically to order) and thus have been unable to determine their auditory capabilities (but see [15]). We identified arthropod prey species from mitochondrial CO1 barcodes recovered from the feces of 51 *B. barbastellus* individuals [16] to investigate the proportion of eared moths in the diet. This genetic approach revealed that *B. barbastellus* is not just a moth specialist; it feeds almost entirely on eared moths (Table 1). The overall proportion of moths in the diet determined by genetic and conventional morphological techniques showed a high level of congruence, providing support for the genetic results (Table 1). In comparison, *N. leisleri* eats few moths (Table 1). Other aerial-hawking bats in the UK feed mainly on flies, with low to moderate amounts of moths in their diets (0 to 36% by volume [14]).

Given that *B. barbastellus* specializes in eating eared prey, we predicted that the echolocation calls of this bat would be inconspicuous to moths. We tracked the flight paths of passing bats in three dimensions with microphone arrays [4, 17] while simultaneously recording neural activity in the auditory nerve of the moth *Noctua pronuba* (Noctuidae; Figure 1), a species commonly eaten by *B. barbastellus* (M.R.K.Z., unpublished data). Noctuid moths possess two auditory sensory cells, A1 and A2. Both are broadly tuned to frequencies above 10 kHz, with A1 being ~20 dB more sensitive than A2 (Figure 2A; [8]). Roeder [18] hypothesized that A1 activity elicits directional flight away from distant bats and A2 elicits an erratic escape response when bats are close. In contrast, other studies on closely related moth families hypothesized that a critical total number of A-cell spikes is sufficient for the latter response [19, 20]. We used two detection criteria in our study: the first occurrence of (1) at least one A1 spike and (2) at least one A2 spike in response to echolocation calls (Figures 1 and 2A). The first occurrence of at least one A2 spike accommodates both previously mentioned hypotheses [18–20]: the total number of A-cell spikes in response to the call that first elicited at least one A2 spike was 11.1 ± 1.8 standard deviations (SDs) (see Supplemental

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Table 1. Percent of Lepidoptera in the Diets of *Barbastella barbastellus* and *Nyctalus leisleri*

Bat species	*Barbastella barbastellus*			*Nyctalus leisleri*
Diet analysis method	Genetic		Morphological	Morphological
Lepidopteran groups	Eared	All	All	All
Percent by volume	85[a]	88[a]	91[a]	56[a]
	-	-	88[b]	10[b]
	-	-	94[b]	12[b]
	-	-	73[b]	0[d]
	-	-	99[b]	10[d]
	-	-	-	20[c]
Median	85	88	91	11

[a]This study.
[b][14].
[c][42].
[d][43].

Experimental Procedures, available online), corresponding with these previous estimates [19, 20] of total spike numbers required to elicit a behavioral response to a nearby bat.

Despite similar calling frequencies for the two bat species, the A1 cell responded to *N. leisleri* calls at 33.2 m or less, whereas it only responded to *B. barbastellus* calls at distances of less than 3.5 m (Mann-Whitney U = 0, p = 0.003, n = 7 and 8, respectively, two-tailed; Figure 2C). The A2 cell responded to *N. leisleri* calls at 18.5 m or less, whereas only one A2 cell response to *B. barbastellus* was recorded, and then at close range (1.8 m, Figure 2C). In seven other moths, *B. barbastellus* came as close as 1.9 m (median, quartiles: 1.9–2.2 m) without eliciting A2 spikes. Likewise, only five of 46 *B. barbastellus* passes elicited more than four spikes per call (maximum of eight spikes in one flight path).

The distance at which a prey animal can detect an approaching predator determines the amount of time available for an escape. At the mean flight speed of 10.3 (±1.7 SD) m/s for *N. leisleri*, the A1 cell of *N. pronuba* will respond ~3.2 s and the A2 cell ~1.8 s before the bat reaches the moth's current position, providing sufficient time to initiate escape responses. In contrast, *B. barbastellus* calls (flight speed: 7.7 [±1.2 SD] m/s) elicit an equivalent neural response only ~0.5 s and 0.25 s before contact, respectively. In addition, reaction times in moths range from 45 to 250 ms [21]. Hence, the high proportion of eared moths in the diet of *B. barbastellus* can be explained by the late detection of the bat by the moth.

To explain why *B. barbastellus* is so inconspicuous to moths, we calculated the source levels (i.e., the call amplitude 10 cm away from the bat's mouth) of search calls for both bat study species based on the call level measured at the microphone and the known distance to the bat. *B. barbastellus* emits two types of calls (Figure 2; [22]). Type 1 is lower in frequency and less frequency modulated than type 2. We only analyzed type 1 calls because the majority of detection distances (92%) were in response to this type, presumably because type 2 calls are emitted upward ([22], U. Marckmann and V. Runkel, personal communication). The median search call source levels were only 94 dB peSPL for *B. barbastellus*, but 127 dB peSPL for *N. leisleri* (see Supplemental Experimental Procedures for peSPL definition). Other aerial-hawking bat species emit calls of similar amplitude to *N. leisleri*, between 121 and 131 dB peSPL (average of 10% loudest calls [4]) and between 114 and 134 dB peSPL (total average [5]). Thus, the calls of *B. barbastellus* are 10–100 times fainter than those of other aerial hawkers.

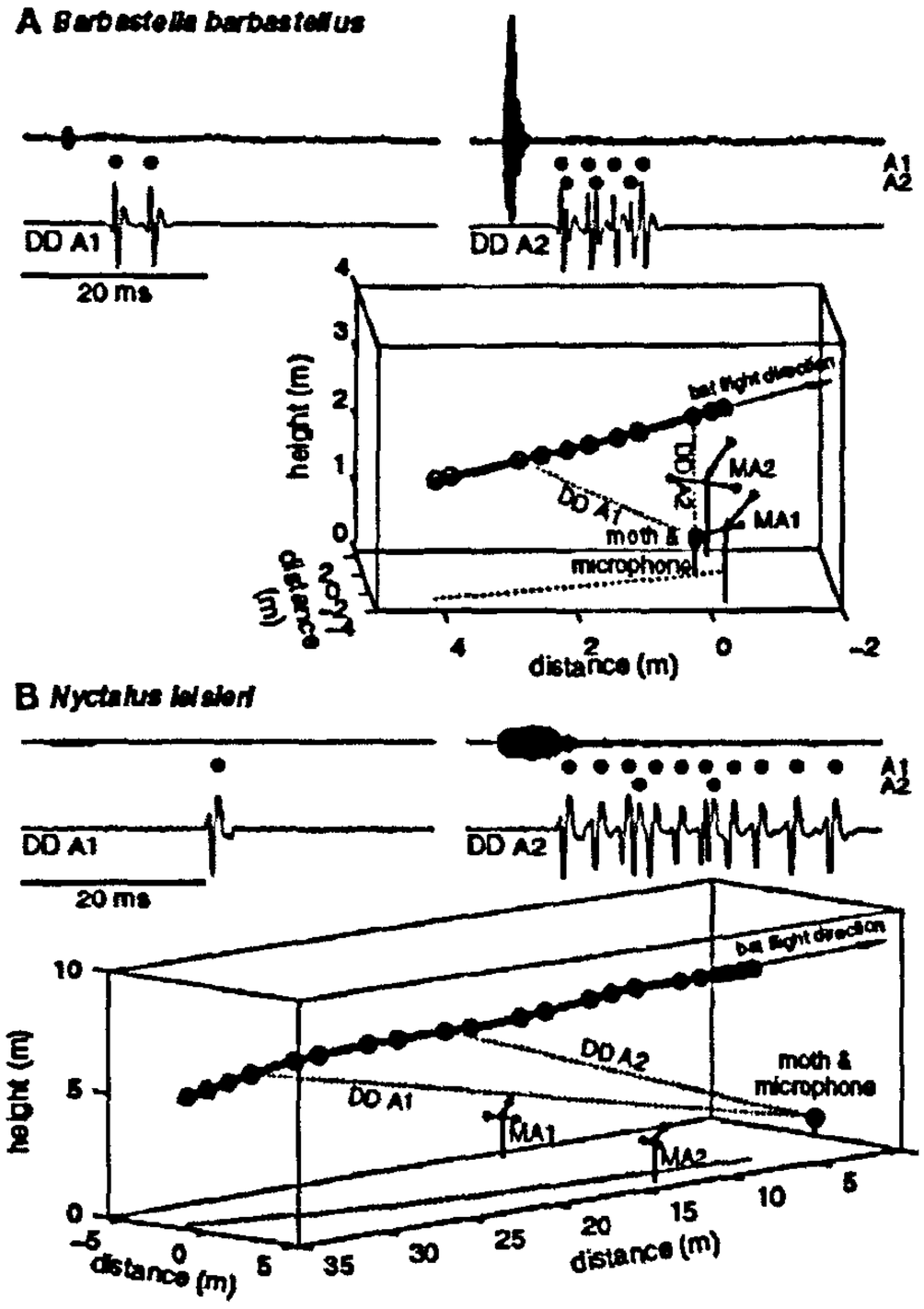

Figure 1. Methods: Acoustic Tracking and Detection Distances

Examples of echolocation calls, elicited moth auditory nerve spike trains, and flight paths of *Barbastella barbastellus* (A) and *Nyctalus leisleri* (B). The top traces show examples of bat echolocation calls (top) and the elicited spike trains (below) of the auditory nerve of the moth *Noctua pronuba* at detection threshold (filled circles, A1 spikes; open circles, A2 spikes). The bottom graphs show the corresponding flight paths. Bat positions were tracked for each emitted call (black circles) with two four-microphone arrays (MA1, MA2). The moth neural preparation and a microphone were placed between or behind the arrays. Maximum detection distances (DDs) were calculated for two detection criteria: first occurrence of at least one A1 spike (DD A1, orange) and at least one A2 spike (DD A2, red).

We developed a perceptual space model that describes the maximum detection distances of bat calls by moths and of moth echoes by bats. The maximum detection distance of a sound source is the distance at which it is just audible. The maximum detection distance of a bat call by a moth is determined by the call's source level, the moth's hearing threshold, and the one-way transmission loss [23]. A bat's maximum detection distance for a moth echo is determined by the call's source level, the bat's hearing threshold, the two-way transmission loss, and the target strength (which is echo attenuation relative to impinging sound) [23]. For the perceptual space model, we calculated maximum detection distances as a function of source level at the median call peak frequencies of 33 kHz (*B. barbastellus*) and 28 kHz (*N. leisleri*). We used the A1 and A2 cell thresholds from audiograms (see Supplemental Experimental Procedures) as moth hearing thresholds, 0 and 20 dB peSPL as bat hearing thresholds [13, 23–26], and a target strength of −16 dB [27]. The transmission loss is one-way for

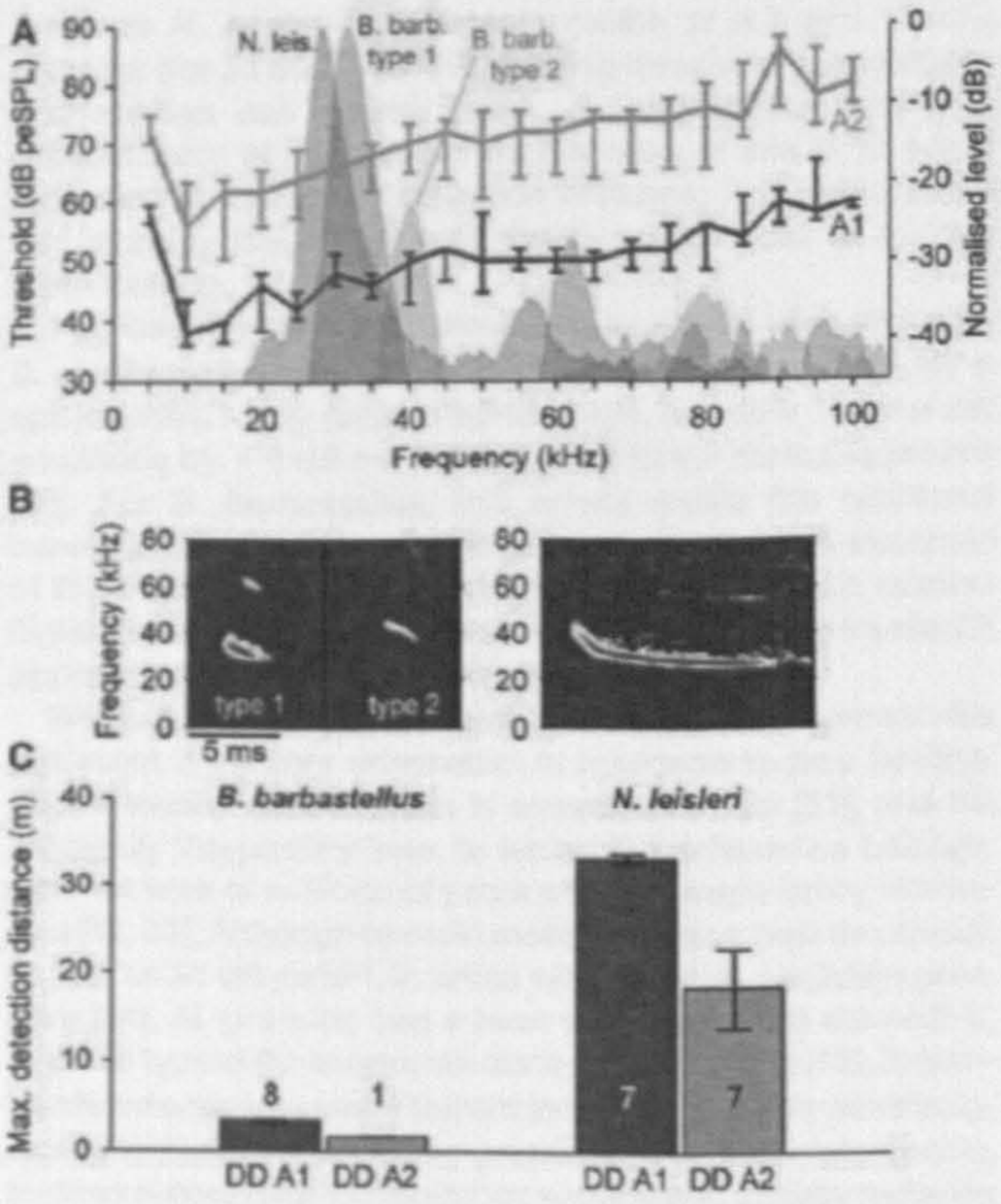

Figure 2. Audiogram and neural Maximum Detection Distances

(A) Audiogram (median ± quartiles, n = 7; left y axis) of the moth *Noctua pronuba* for two different detection criteria (c.f. Figure 1). Colored shadings show normalized spectra of bat calls (right y axis).

(B) Spectrograms of typical calls of the bats *Barbastella barbastellus* and *Nyctalus leisleri*.

(C) Neural maximum detection distances of the moth *N. pronuba* (medians of the individual moth medians ± quartiles) for the bats *B. barbastellus* and *N. leisleri* measured in the field for two different detection criteria (DD A1 and DD A2, c.f. Figure 1). Numbers: sample size of moths showing a neural response.

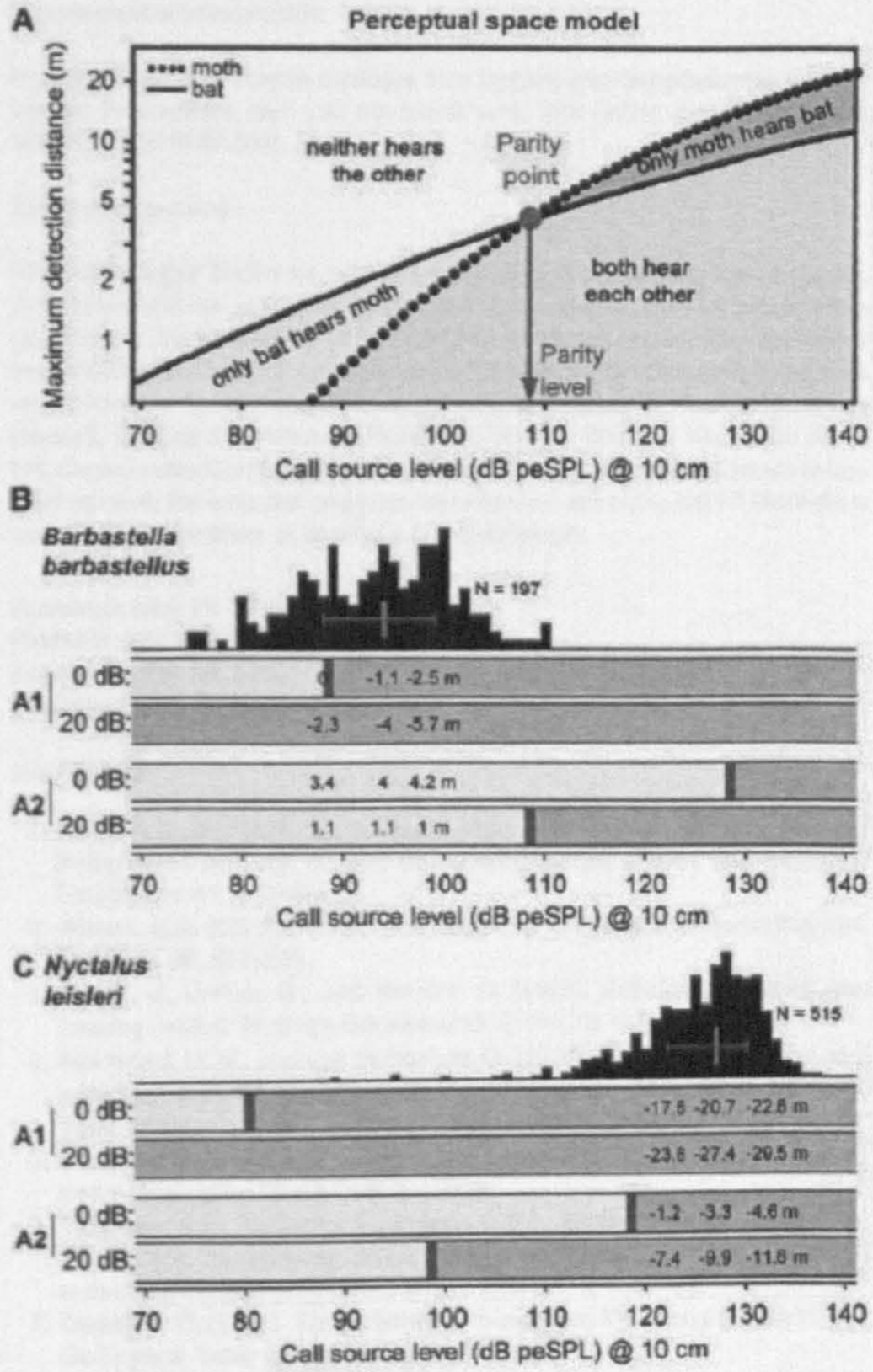

Figure 3. Perceptual Space Model

(A) At low call source levels, bats hear moth echoes over greater distances than moths hear bat calls, and vice versa at high source levels.

(B and C) Measured call source levels (top histograms) and model classification as "bat wins" (yellow) and "moth wins" (blue) for calls of *B. barbastellus* (B) and *N. leisleri* (C). The model classification is given for the moth's A1 and A2 cell thresholds, and for bat hearing thresholds of 0 dB and 20 dB SPL. Differences in detection distance are stated for the marked median and quartiles of the source-level distribution (gray lines). Figure S1 shows the full models of each threshold combination. Figures S2 and S3 show that the stealth echolocation of *B. barbastellus* is effective for all relevant climatic conditions and moth target strengths.

the moth but two-way for the bat. Bats, however, have lower hearing thresholds than moths. Therefore, a source level exists with equal detection distance for bat and moth, which we term the parity level (Figure 3A). At call source levels above the parity level, the moth detects an approaching bat first ("moth wins"), whereas the bat detects the moth first at lower levels ("bat wins," Figure 3A). Figures 3B and 3C classify different call source levels into "bat wins" and "moth wins" for *B. barbastellus* and *N. leisleri* for all four combinations of the two moth auditory cells (A1 and A2) and two potential bat hearing thresholds (0 dB and 20 dB SPL).

The predicted detection distances of bat calls by moths (*N. leisleri*: A1: 35.6 m [quartiles: 34.4–36.6 m], A2: 22.0 m [quartiles: 18.6–22.6 m]; *B. barbastellus*, A1: 4.3 m [quartiles: 3.4–6.1 m]) match our field-measured detection distances (Wilcoxon paired sample test; *N. leisleri*: A1: 33.2 m [quartiles: 32.8–33.8 m], $p = 0.078$, $T = 3$, $n = 7$; A2: 18.5 m [quartiles: 13.8 – 22.8 m], $p = 0.453$, $T = 9$, $n = 7$; *B. barbastellus*, A1: 3.5 m [quartiles: 3.3 – 3.5 m]; $p = 0.102$, $T = 6$, $n = 8$). For *N. leisleri*, the model shows that at all measured call source levels, A1 always, and A2 predominantly, reacts before the bat hears the moth echoes (Figure 3C). In contrast, for *B. barbastellus*, A1 reacts often, but A2 never, before the bat hears the moth (Figure 3B). Given the current hypotheses for the neural code for evasive flight outlined above [18–20], this, as well as the low number of elicited spikes per call, suggests that directional flight might be elicited by *B. barbastellus*, but evasive flight is unlikely. The bat's detection distance of moths is 1.0–1.1 m larger than the A2 detection distance of bats at 20 dB SPL bat hearing threshold and 3.4–4.2 m at 0 dB SPL bat hearing threshold (measured between the quartiles of the call amplitude distribution, Figure 3B). Therefore, *B. barbastellus* uses a stealth echolocation strategy by emitting low-amplitude calls that exploit the relative difference in hearing thresholds between predator and prey, a strategy previously suggested by Fenton and Fullard [28] and by Surlykke [29] and now supported with field-based measurements. This strategy, however, comes at the cost of reduced prey detection distance.

Whereas *N. leisleri* first detects moths at 8.7 and 15.4 m distance (for 20 and 0 dB SPL hearing threshold, respectively, and median call source level), *B. barbastellus* only first detects them at 2.2 and 5.0 m. Because of this 3- to 4-fold reduction in maximum detection distance, it is unlikely that this strategy has benefits to the bat other than to counter moth hearing.

Yet, despite being imperceptible to moths at a distance, *B. barbastellus* should eventually become audible as it approaches. Many aerial-hawking bats, however, reduce call amplitude by ~6 dB per halving of distance during approach [30]. For *B. barbastellus*, this would create the additional benefit that call amplitude remains below detection threshold of the moth's A2 cell, presumably preventing erratic evasive flight (Figure S4). In this way, the bat could maintain its stealth approach from initial detection until capture.

We suggest that the low-amplitude calls of *B. barbastellus* represent a sensory adaptation in response to prey hearing. High-intensity echolocation is ancestral in bats [31], and the subfamily Vespertilioninae, to which *B. barbastellus* belongs, evolved tens of millions of years after the moth family Noctuidae [32, 33]. Although noctuid moths can have best thresholds as low as 20 dB peSPL in areas with high bat predation pressure [34], *N. pronuba* has a best threshold of 38 dB peSPL, which is typical for temperate-zone noctuid moths [13]. In addition to selective pressure toward increased auditory sensitivity, moths experience opposing pressure from associated costs, such as responding to innocuous sounds and thereby reducing their feeding and mating opportunities, and possibly risking predation after landing on a surface [35]. It thus appears that the rarity of this predator strategy did not provide sufficient selection pressure for an adaptive response in moths (the rare-enemy effect [7]; c.f. [36]), as also suggested for the gleaning bat *Myotis septentrionalis* [12] and the low-frequency bat *Euderma maculatum* [37]. As a result, *B. barbastellus* currently has a major advantage in the predator-prey arms race and can avoid competition with bats that emit louder calls. Sensory differences, like the example presented here, play an important role in determining access to food, reducing competition between coexisting species and structuring communities [38, 39].

Experimental Procedures

B. barbastellus diet composition (n = 51 individual bats) was assessed by DNA barcoding [16] and morphological methods [40]. We tracked three-dimensional flight paths of wild free-flying bats by using time-of-arrival differences of their echolocation calls between microphones on two four-microphone arrays [4, 17]. We calculated the distance of bats from a moth auditory preparation placed between or behind the arrays. Neural activity was recorded from the auditory nerve of the noctuid moth *Noctua pronuba*. We used two criteria for determining maximum detection distances: the first responses of the A1 or A2 sensory cells. We recorded the echolocation calls of bats with a calibrated microphone placed next to the moth and calculated source levels (dB peSPL re. 20 μPa at 10 cm distance from the bat's mouth) of search calls. We obtained audiograms of seven individuals of the moth *N. pronuba* for the same detection criteria as used in the field by playing back pure-tone pulses of 20 ms duration at increasing amplitudes from 20 to 90 dB peSPL in the laboratory. We calculated maximum detection distances for the perceptual space model by using the sonar equation [23] for −16 dB target strength [27], 18°C temperature, 70% relative humidity (average climatic conditions of our recording nights), and 28 and 33 kHz call peak frequencies. We used bat hearing thresholds of 0 and 20 dB SPL [13, 23–26]. Moth hearing thresholds for the A1 and A2 cells were retrieved from the audiograms at 28 and 33 kHz and corrected for the shorter call duration compared to audiogram pure tones [41]. Full methods are available in the Supplemental Information.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.07.046.

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